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14. ABSTRACT

About one-third of patients with cancer have metastases that are detected at the time their cancer is first diagnosed, and an additional third of patients have metastases that are too small to be detected by usual diagnostic tests. These micrometastases, however, will eventually grow into clinically significant metastases if the patient receives no treatment or local treatment of the primary tumor only. Metastatic breast cancer is a disease with low survival rates. The treatment of metastatic breast cancer involves the palliation of symptoms by reducing the tumor's size and growth rate. Currently, the effectiveness of therapy is determined by a series of tests. Imaging tests may include bone scan, radiograph, magnetic resonance imaging (MRI), and computed tomography (CT). Blood tests may include tumor markers, such as CA 27.29 and CA 15-3. The detection of circulating tumor cells has been proposed as a method to assess response to treatment of metastatic breast cancer. The detection of tumor cells may have clinical utility in risk stratification in early breast cancer, in early detection of relapse, and in monitoring the response to treatment. The circulating cells appear to have characteristics of tumor cells and may be identified in the peripheral blood of patients with cancer. The techniques that have been used to detect circulating tumor cells include cytometric and nucleic acid based approaches. The cytometric approaches use immunocytochemical methods to identify and characterize the individual tumor cells. Nucleic acid-based approaches detect the DNA and RNA sequences that are differentially expressed in tumor cells and normal blood components. The purpose of this study is to determine whether WAVE3, a metastasis promoter gene, can be used as a predictive marker for the progression and metastasis of breast cancer, using immunohistochemistry on human breast cancer tumors of different stages of breast cancer. An additional objective is to determine whether WAVE3 expression levels in the blood of breast cancer patients may have prognostic value after the administration of adjuvant chemotherapy in women with operable breast cancer. 14. ABSTRACT The first year of this study focused on identification and preliminary characterization of the tumor specimens and corresponding control samples. The Principal Investigator (PI) successfully identified most of the 100 tumor specimens (estrogen receptor [ER]-negative and histological grade III) with the most complete and informative clinical and pathological data from the Roswell Park Cancer Institute (RPCI) Tumor Registry. An additional cohort of 100 ER+ and histological grade I tumors is currently being identified.

15. SUBJECT TERMS
Breast cancer, Circulating Tumor Cells, WAVE3, Immunostaining, Metastasis

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Introduction

Cancer metastasis is a complex process, usually requiring cancer cells to escape from the primary site, survive in the blood/lymph system and then have the ability to establish at a distant site. We have shown that the expression levels of WAVE3, an actin polymerization protein, are significantly correlated with advanced stages of breast cancer, using immunohistochemistry analysis. Our preliminary data also suggest that the ER^- tumors, which are believed to be associated with a poor prognosis, show the highest levels of WAVE3 staining. Together with our published data on the role of WAVE3 in breast cancer progression and metastasis, we hypothesize that WAVE3 may function as a metastasis promoter gene.

On the other hand, breast cancer is considered a systemic disease because early tumor cell dissemination may occur even in patients with small tumors. The association between the presence of circulating tumor cells (CTCs) in the blood of patients with metastatic carcinoma and short survival is gaining increased support from the findings of several studies. Furthermore, the presence of CTCs after adjuvant chemotherapy has been associated with a poor clinical outcome in patients with early-stage breast cancer. Moreover, the detection of tumor cells-specific biomarkers in the blood before and after the adjuvant systemic treatment could help to identify those patients who may have a substantial clinical benefit from a 'secondary' adjuvant treatment before the occurrence of overt metastasis.

We, therefore, proposed the following specific Aims

Specific Aim 1: To test whether WAVE3 levels can be used as a predictive marker for the progression and metastasis of breast cancer.

Specific Aim 2: To evaluate the prognostic value of WAVE3 expression levels in circulating tumor cells after the administration of adjuvant chemotherapy in women with operable breast cancer.

Body

Specific Aim 1: To test whether WAVE3 levels can be used as a predictive marker for the progression and metastasis of breast cancer.

1.A. Performance of Tasks proposed in the SOW

Task 1: Specimen identification

We identified the tumor specimens for which the most complete and informative clinical and pathological data is available.

Approximately 400 women undergo treatment for primary breast cancer each year at the RPCI Breast Center each year. A database of these patients has been maintained since 1995 by a professional data manager that contains pertinent clinical, pathological and treatment information on each patient. Recurrence and survival data is maintained by the RPCI Tumor Registry and can be linked to the breast cancer database. For this study, we were able to identify **64 out of 100** proposed women who are ER negative and histologic grade III based on the standard Scarff-Bloom-Richardson grading system. We were able to match them with **64 out of 100** proposed women who are ER+ and grade 1

Quality control was performed for all the tumors to determine their suitability for the slide preparation and immunohistochemical (IHC) staining.

Task 2: Slide preparation and staining were performed according to what was proposed in the SOW:

Slide preparation and staining: At least 4 slides will be prepared from each tumor, and the quality of slides will also be assessed and only the slides that pass the quality control, as judged by our expert pathologists, will be used for the subsequence staining procedures.

For each tumor we will perform the following stainings:

1) H&E to confirm the presence of tumor tissue and to determine the extent of tumor invasiveness.

2) Staining for the WAVE3 protein using a polyclonal rabbit anti-WAVE3 antibody, which has already been confirmed as suitable for IHC staining (Sossey-Alaoui et al., 2007).

3) Staining with rabbit IgG as negative control and to determine the back ground level of the staining.

Task 3: Tissue microarrays (TMA) preparation.

Since we were very satisfied with the quality of staining of the individual slides we determined that the TMA preparation has not become a high priority as least for the time being. We will however keep this task on the to do list.

Task 4: Scoring of the staining and Data analysis were conducted according to the SOW:

As stated above we have performed most of the Tasks for specific Aim 1 according to the time frame proposed in the SOW.

1.A. BACKGROUND

WAVE proteins coordinate actin cycling and cell motility.[1] The WAVE3 subtype is constitutively expressed in metastatic human breast cancer cell lines.[2] Its induction and activation results in membrane changes that enhance breast cancer cell migration.[2] Blockade of WAVE3 transcription disrupts breast cancer cell migration.[2] An association between WAVE3 over-expression and decreased estrogen receptor (ER) expression and histologic grade has been suggested.[3] WAVE3 expression is associated with enhanced breast cancer cell migration and adverse tumor features and, therefore, may be associated with the acquisition of metastatic potential in high risk tumors.

1.B. OBJECTIVE

The association between WAVE3 and ER status and histologic tumor grade was studied. WAVE3 expression and its association with the development of distant recurrence was also examined.

1.C. METHODS

Our institutional breast cancer (BC) database was reviewed for patients who presented with invasive BC from 1999-2009. A matched cohort design was utilized. Matching by pathologic stage and treatment was achieved for 61 patients with Scarff-Bloom-Richardson (SBR) grade 1 and ER+ tumors (SBR1/ER+) to 61 patients with SBR grade 3 and ER- tumors (SBR3/ER-). Cytosolic WAVE3 expression was determined by immunohistochemistry. The product of stain intensity (0-3) and percentage of cells staining (0-100) was used to derive a WAVE3 score (0-300). The WAVE3 score between each cohort was compared and the association between WAVE3 score and a variety of clinico-pathologic features was examined. The log rank test was used to compare distant recurrence free survival at various WAVE3 scores. A score of $\geq$212 was found to have the strongest association with distant recurrence and was used as a positive threshold for subsequent survival analyses.

Analysis of categorical data between two groups was performed with the χ^2 square test. Analysis of continuous variables for two groups was with the Mann-Whitney rank sum or the Pearson product moment correlation. Analysis of continuous variables for more than two groups was with the Kruskal-Wallis one way ANOVA . Survival was analyzed using the Kaplan-Meier method and compared using the log-rank test. The multiple linear regression method was performed to compare survival as a function of patient and treatment factors.

1.D. RESULTS

Table 1. Cohort clinical and tumor characteristics			
Variable	**SBR1/ER+ (n=61)**	**SBR3/ER- (n=61)**	**p**
Age at Diagnosis (years)	**57**	**57**	**0.479**
Tumor Size (cm)	**1.5**	**1.9**	**0.136**
Lymph Node Status			**0.714**
Negative	**34 (56%)**	**36 (59%)**	
Positive	**27 (44%)**	**25 (41%)**	
TNM Stage			**0.981**
Stage I	**22 (36%)**	**21 (34%)**	
Stage II	**31 (51%)**	**32 (53%)**	
Stage III	**8 (13%)**	**8 (13)%**	
Her2 Status			**<0.001**
Negative	**56 (92%)**	**41 (67%)**	
Positive	**3 (5%)**	**20 (33%)**	
Frequency of recurrence			**<0.001**
All recurrence	**1 (2%)**	**16 (26%)**	
Local recurrence	**1 (2)%**	**6 (10%)**	
Distant recurrence	**0 (0%)**	**10 (16%)**	
Disease specific mortality	**0 (0%)**	**7 (11%)**	**0.006**

Table 1. Increased Her-2 neu receptor expression, distant recurrence and breast cancer related mortality were seen in the SBR3/ER- cohort compared to the SBR1/ER+ cohort (33% vs 5%, $p<0.001$, 16% vs. 0%, $p<0.001$, and 11% vs. 0%, $p=0.003$, respectively).

Table 2. Association of WAVE3 score with ER status and SBR grade			
Variable	**SBR1/ER+ (n=61)**	**SBR3/ER- (n=61)**	**p**
Median WAVE3 score	**160**	**180**	**0.703**

Table 2. There was no difference in WAVE3 score between the two matched cohorts. (SBR1/ER+, 160 vs. SBR3/ER- ,180, p=0.703).

Table 3. Association between WAVE3 score and tumor features			
	Median WAVE3 score		
Variable	**All patients (n-122)**	**SBR1/ER+ (n=61)**	**SBR3/ER+ (n=61)**
Tumor size	**[a]0.234, p=0.009**	**0.201, p=0.120**	**[a]0.261, p=0.042**
Lymph Node Status			
Negative	**145**	**130**	**170**
Positive	**200**	**200**	**180**
TNM Stage			
Stage I	**160**	**140**	**200**
Stage II	**180**	**200**	**150**
Stage III	**240**	**190**	**255**
Her2 neu status			
Negative	**180**	**160**	**180**
Positive	**200**	**200**	**200**

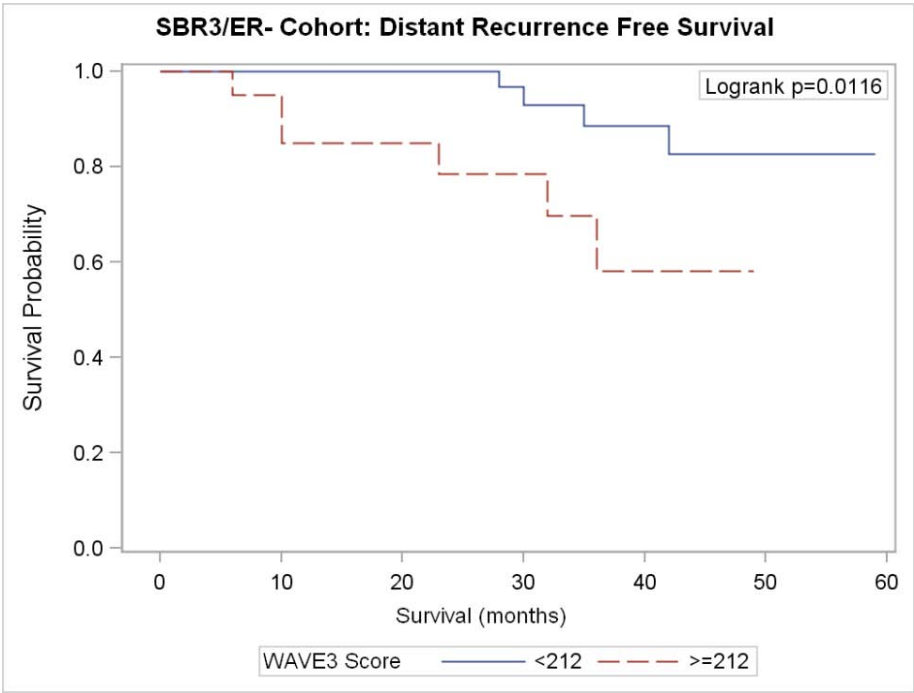
Table 3. Median WAVE3 score increased with tumor size for the entire study group (Pearson correlation 0.234, p=0.009), but only remained significantly associated for the SBR3/ER- cohort (Pearson correlation 0.261, p=0.042).
Median WAVE3 score increased with lymph node status (positive 200 vs. negative, 145, p =0.034), but only remained significantly associated for the SBR1/ER+ group (positive 130 vs negative 200, p=0.023).
Median WAVE3 score increased with pathologic stage (I, 160 vs. II,180 vs III, 240, p=0.012) but only remained significantly associated for the SBR3/ER- group (I, 200 vs II, 150 vs II, 255, p=0.006).
WAVE3 score and Her2-neu receptor status were not associated (Her2neu- 180 vs. Her2neu+ 200, p=0.509).

Table 4. Association between WAVE3 score and outcome			
	Median WAVE3 score		
Variable	All patients (n-122)	SBR1/ER+ (n=61)	SBR3/ER+ (n=61)
Distant recurrence			
No	NA	NA	160
Yes	NA	NA	240
Disease specific mortality			
No	NA	NA	170
Yes	NA	NA	270

Table 4. Only patients in the SBR3/ER- cohort experienced distant recurrence or disease specific mortality. WAVE3 scores were higher for patients with either adverse clinical outcome

1.B.1. Low staining of WAVE3 correlates with overall disease free survival.

We found a very significant correlation between the levels of WAVE3 staining in the primary tumors and the overall disease free survival. Those patients with low levels of WAVE3 staining in the primary tumors were found to live longer disease free after the primary tumors was removed compared to those patients with high levels of WAVE3 staining.



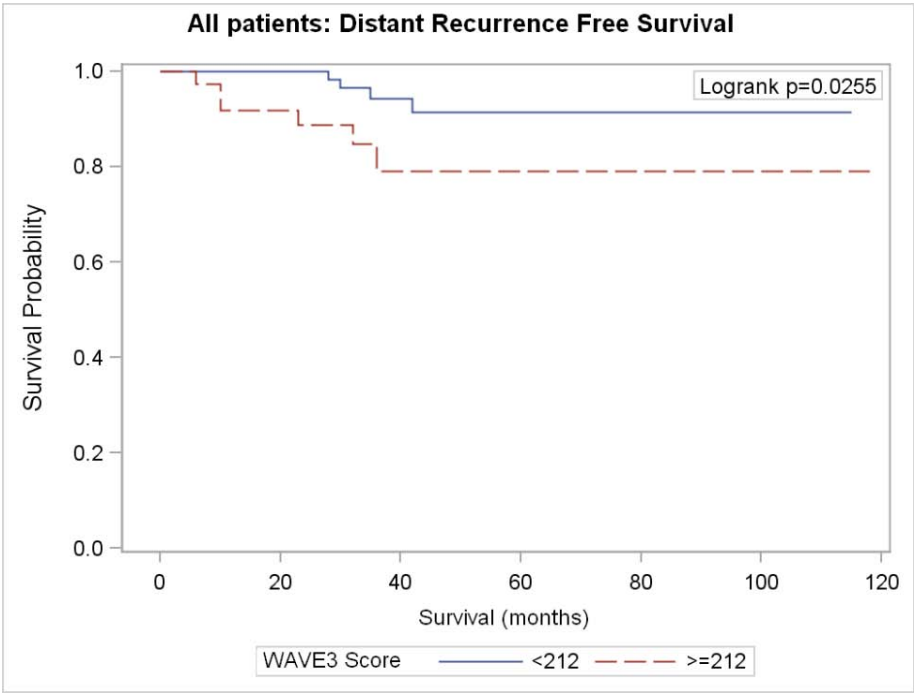
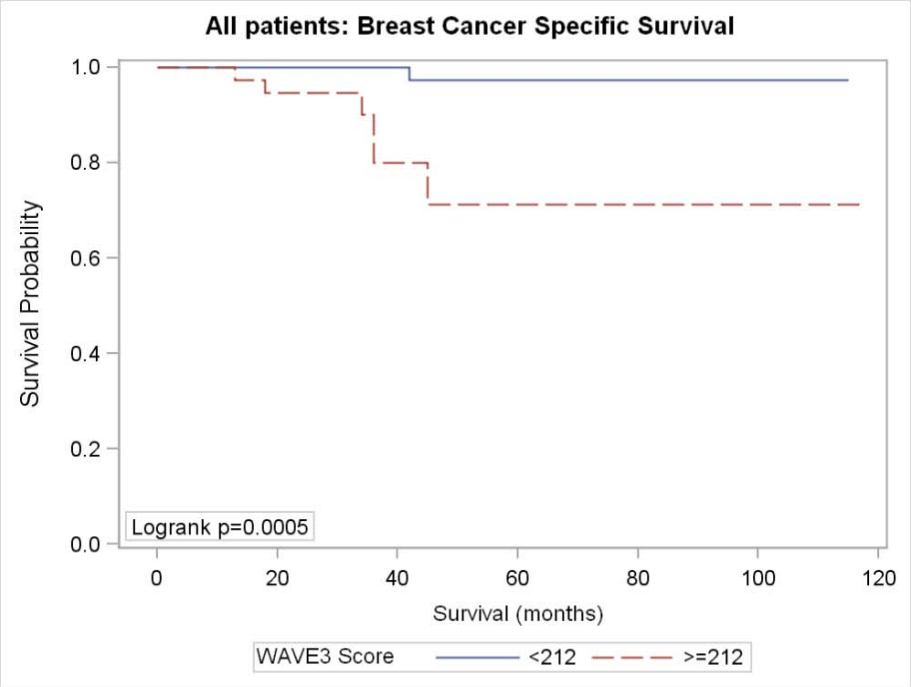


Figure 1. WAVE3 score ≥212 was significantly associated with decreased distant recurrence free survival in the entire study group and the SBR3/ER- cohort (p=0.0255 and p=0.0156, respectively).

1.B.2. The recurrence free survival is tumor grade-dependent.

We also found that a very significant correlation between the tumor grade and the overall disease free survival. Patients with grade 1 tumors and low WAVE3 staining tend to live longer with no detectable disease compared to those patients with grade 3 tumors and high levels of WAVE3 staining.



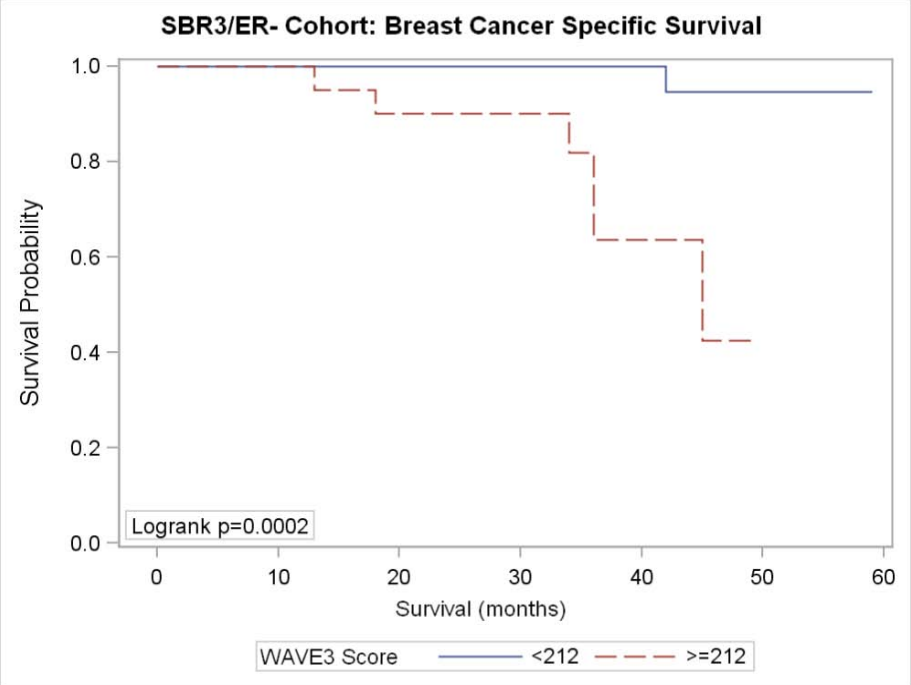


Figure 2. WAVE3 score ≥212 was significantly associated with decreased breast cancer specific survival in the entire study group and the SBR3/ER- cohort (p=0.0005 and p=0.0002, respectively

On multivariate analysis a WAVE3 score ≥212 did not remain independently associated with distant disease free survival (p=0.062) but was independently associated with an increased risk for breast cancer specific mortality (p=0.009).

1.B.3. Correlation between the WAVE3 staining levels and distant metastasis.

One of the very significant finding is that WAVE3 staining levels can be used an independent marker for survival recurrence as well as for disease-specific mortality risk.

1.B.4. CONCLUSIONS.

An association between ER status, SBR grade and WAVE3 score was not verified. WAVE3 score is associated with tumor size, lymph node status, and pathologic stage. Patients in the SBR3/ER- group were more likely to develop distant recurrence and disease specific mortality. A WAVE3 score $\geq$ 212 was associated with breast cancer specific survival on uni - and multivariate analysis. WAVE3 score may be able to predict adverse outcome in high risk breast cancer patients.

Specific Aim 2: Evaluate the prognostic value of WAVE3 expression levels in circulating tumor cells after the administration of adjuvant chemotherapy in women with operable breast cancer.

2.A. Performance of Tasks proposed in the SOW

Task 5: We proposed to identify the blood specimens for which the most complete and informative clinical and pathological and outcome data is available.
We will analyze 100 samples from Stage I and/or II breast cancer patients, and 100 samples from healthy females without cancer history.

We have identified all the samples described above which were linked to complete clinical and pathological and outcome data. The blood samples were shopped to Cleveland Clinic about three months ago.

Task 6: We proposed to:
Determine the presence or absence of WAVE3 mRNA in the blood of breast cancer patients before and after chemotherapy. The blood from subjects with no evidence of disease will be used as controls. We will:

a) Prepare total RNA from the blood specimens using standard RNA extraction protocols.
b) Assess the quality of the RNA by RNA-Agarose gel electrophoresis.
c) Use β–Actin and other internal controls such as WAVE2 (WAVE2 is ubiquitously expressed in the white blood cells) to ensure the quality of the RT-PCR and as loading controls.
d) Perform Nested RT-PCR and agarose gel electrophoresis to determine the presence or absence of WAVE3 transcripts in the blood specimens, and record the results.
e) Repeat tasks b to d at least 3 times to insure reproducibility of the results.
f) Repeat tasks a to e for the specimens with questionable results.

Completed. See below

Task 7: A BC TMA is being Built that contains more than 120 BC specimens from different stages and genetic subtype.

Task 8: Underway

2.B. Background.

WAVE3 is expressed at very low levels in the hematopoietic cells, but its expression levels are higher in the circulating tumor cells (CTC) as a result of early tumor cell dissemination even in patients with small tumors. We used this WAVE3-specific characteristic to evaluate the prognostic value of WAVE3 expression levels in CTCs in women with operable breast cancer. A second cohort consisted of 200 BC patients from whom blood was collected before surgery and

initiation of therapy. Quantitative real-time RT-PCR was utilized to determine the expression levels of WAVE3 in total RNA extracted from the circulating tumor cells. The WAVE3 score between each subtype was compared and the association between WAVE3 score and a variety of clinico-pathologic features was examined

2.C. Methods.

Total RNA was extracted from each sample's Buffy coat using TRIzol reagent (Invitrogen), following to the manufacturer's instructions. cDNA was generated and used as a template for semi-quantitative RT-PCR performed as previously described (5;8;14;15). Expression levels of microRNAs were quantified by real-time quantitative RT-PCR using the human TaqMan MicroRNA Assays Kits (Applied Biosystems). The reverse transcription reaction was carried out with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. For each gene, quantification of expression levels was performed using the respective gene-specific primers (Table S2) and the RT^2 SYBR Green/Fluorescein qPCR Master Mix (SABiosciences) following the manufacturer's instructions. Quantitative PCR was performed on the BioRad iCycler PCR system where the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The cycle threshold (Ct) values were calculated with SDS 1.4 software (Bio-Rad). The expression levels of each transcript were normalized using the $2^{-\Delta\Delta Ct}$ method (16) relative to GAPDH. The ΔCt was calculated by subtracting the Ct values of GAPDH from the Ct values of the transcript of interest. The ΔΔCt was then calculated by subtracting ΔCt of the matching normal human breast tissue from the ΔCt of cancer tissues, or the ΔCt of MCF10A cell line for the established cancer cell lines. Fold change in the gene was calculated according to the equation $2^{-\Delta\Delta Ct}$.

The WAVE3 expression levels (score) between each cohort was compared and the association between WAVE3 score and a variety of clinico-pathologic features was examined. The log rank test was used to compare distant recurrence free survival at various WAVE3 scores. A score of ≥212 was found to have the strongest association with distant recurrence and was used as a positive threshold for subsequent survival analyses.

Analysis of categorical data between two groups was performed with the χ^2 square test. Analysis of continuous variables for two groups was with the Mann-Whitney rank sum or the Pearson product moment correlation. Analysis of continuous variables for more than two groups was with the Kruskal-Wallis one way ANOVA . Survival was analyzed using the Kaplan-Meier method and compared using the log-rank test. The multiple linear regression method was performed to compare survival as a function of patient and treatment factors.

2.D. Results

2.D.1. WAVE3 transcripts can easily be detected in the blood of metastatic breast cancer patients and not in the normal blood.

We have conducted a pilot study as a proof of principle to determine whether WAVE3 can be detected in the blood of metastatic cancer patients. We randomly chose 10 blood samples (from metastatic breast cancer patients) that are part of the archived blood repository, which were matched with 10 blood samples from healthy controls without any cancer history. The Nested-RT-PCR assay showed that while no WAVE3 mRNA could not be detected in any of the control blood samples without cancer history, different levels of WAVE3 mRNA was amplified from all six blood specimens belonging to patients with metastatic breast cancer (Figure 1).

These preliminary data, while they confirm the sensitivity of our assay (Figure 1), also provide a preliminary demonstration that WAVE3 could be used as a biomarker for early follow-up on the progression and relapse of metastatic cancer.

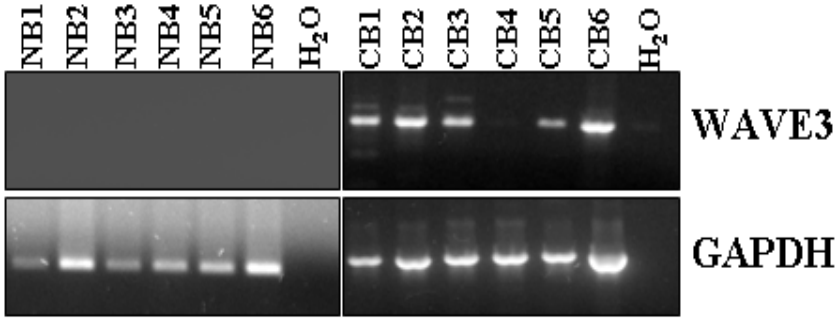


Figure 1: Nested RT-PCR from total RNA extracted from blood cells of six healthy control individuals and from the blood cells of six randomly chosen patients with metastatic cancer. WAVE3 mRNA could be amplified from the breast cancer patients, but not form from the healthy controls. GAPDH was used as an internal control for the integrity of the RNA and as an equal loading control.

2.D.2. COHORT CHARACTERISTICS

Variable	N	N Missing	Min	25%	Median	75%	Max	Mean	Std
WAVE3.RT.Score	199	1	0.5	10.3	23.54	43.39	99.54	29.84	23.57
Age At Draw	200	0	23	43	53	62.25	88	53.56	13.35
T Size	193	7	0.01	0.9	1.5	2.3	11	1.84	1.55
Nodes Resected	199	1	1	3	6	14	42	9.48	8.95
Nodes Positive	199	1	0	0	0	1	27	1.41	3.43
Days To Recurrene	9	191	0	236	421	671	804	431	281.16
Age At Recurrence	9	191	32	35	54	56	79	51.44	16.8

Other patients chacateristics:

Variable	level	N	percentage
SampleStatusme	No prior treatment (excluding diagnostic biopsy)	159	79.5
	Post-surgical/No adjuvant or systemic therapy	41	20.5
Overnight	No	200	100
CaseControl	Case	200	100
Sex	Female	200	100
Race	Black	26	13
	White	174	87
ParticipantAttributeTypeDesc	New (Distant)	1	0.5
	New (In Situ)	5	2.5
	New (Localized)	128	64
	New (Regiol)	66	33
FilSurg	Lumpectomy	131	65.5
	Total mast w/immediate reconstruct	32	16
	Total mast w/no immediate reconstruct	37	18.5
AxilStage	Axil dissection level I/II (lumpectomy or MRM)	66	33.2
	Axil dissection level I/II/III	1	0.5
	Sentinel Node biopsy only	132	66.3
SizeNotation	Multicentric	1	3.6
	Multifocal	8	28.6
	Neoadjuvant	18	64.3
	Not reported	1	3.6

ClinicalStg	I T1 N0 M0	1	5.6
	IIA T1 N1 M0	1	5.6
	IIA T2 N0 M0	6	33.3
	IIB T2 N1 M0	4	22.2
	IIB T3 N0 M0	1	5.6
	IIIA T3 N1 M0	1	5.6
	IIIB T4 Any N M0	2	11.1
	IIIB T4 N1 M0	2	11.1
Stage	I T1 N0 M0	94	47
	IIA T1 N1 M0	25	12.5
	IIA T2 N0 M0	31	15.5
	IIB T2 N1 M0	20	10
	IIB T3 N0 M0	2	1
	IIIA T1 N2 M0	5	2.5
	IIIA T2 N2 M0	8	4
	IIIA T3 N1 M0	4	2
	IIIA T3 N2 M0	1	0.5
	IIIB T4 Any N M0	2	1
	IIIB T4 N1 M0	2	1
	IIIC Any T N3 M0	6	3
ER	Negative	96	48
	Positive	104	52
PR	Negative	101	50.5
	Positive	99	49.5
Her.2	Negative	166	83.8
	Strong	31	15.7
	Weak	1	0.5
NuclearGrade	I	22	11.7
	II	56	29.8
	III	110	58.5
VascLymphaInvas	No	132	72.9
	Yes	49	27.1
Necrosis	No	135	71.8
	Yes	53	28.2
Bilateral	FALSE	190	95
	TRUE	10	5
MenopausalStat	"2 = Yes, menstrual periods on hormone replacement therapy"	2	1
	tural Periods	75	37.5
	No Periods	123	61.5
ClinT	T1	1	0.5
	T1a	1	0.5
	T1b	39	19.5
	T1c	70	35
	T2	49	24.5
	T3	8	4
	T4d	4	2
	Tis	9	4.5
	Tx	19	9.5
ClinN	N0	171	85.5
	N1	22	11
	N2	2	1
	Nx	5	2.5
PathT	T1a	16	8.7

	T1b	32	17.5
	T1c	73	39.9
	T1mic	3	1.6
	T2	51	27.9
	T3	6	3.3
	T4d	1	0.5
	Tx	1	0.5
PathN	pN0	116	63.4
	pN0(i+)	1	0.5
	pN0(i-)	2	1.1
	pN1a	35	19.1
	pN1c	1	0.5
	pN1mi	7	3.8
	pN2a	15	8.2
	pN3	2	1.1
	pN3a	4	2.2
Grade	I (well diff.)	8	4.2
	II (mod. diff.)	36	18.8
	III (poorly diff.)	147	77
Histology	Ductal clinical inflammatory	2	1
	Ductal inflammatory (w/path dermal lymph invasion)	1	0.5
	Ductal invasive, NOS	125	62.5
	Ductal papillary	1	0.5
	Invasive Mixed Ductal and Lobular	3	1.5
	Invasive w/predomint intraductal component	1	0.5
	Invasive with predomint intraductal component	48	24
	Lobular invasive	18	9
	Metaplastic	1	0.5
FirstRecurSite	Bone	1	11.1
	Ipsilateral breast	4	44.4
	Liver, parenchyma	2	22.2
	Skin	2	22.2
X1stRecurTx	Arimidex	1	12.5
	Bevacizumab (Avastin)	1	12.5
	Capecitabine	2	25
	Gemcitabine-Albumin-bound Paclitaxel (Abraxane)	1	12.5
	Gemcitabine-Capecitabine	1	12.5
	Zolendrote (Zometa)	1	12.5
	Zometa-Lapitinib	1	12.5
ChemoType	Doxorubicin (Adriamycin, Adriamycin-TM)	1	0.8
	AC	3	2.3
	AC-Docetaxel-Herceptin	1	0.8
	AC-Herceptin	1	0.8
	AC-Paclitaxel	7	5.5
	AC-Paclitaxel-Bevacizumab (Avastin)	1	0.8
	AC-Paclitaxel-Herceptin	7	5.5
	AC-Taxol-Herceptin	1	0.8
	AC-Taxotere	1	0.8
	CT (Cyclophosphamide(Cytoxan)/Paclitaxel (Taxol)	2	1.6
	Clinical Trial Drug--nonblinded	1	0.8
	Cyclophosphamide (Cytoxan, CTX)-Albumin-	1	0.8

	bound Paclitaxel (Abraxane)		
	Cyclophosphamide(Cytoxan)/Docetaxel(Taxotere)	13	10.2
	Cyclophosphamide(Cytoxan)/Docetaxel(Taxotere)-Clodrote	1	0.8
	Cyclophosphamide(Cytoxan)/Docetaxel(Taxotere)-Herceptin	2	1.6
	Docetaxel (Taxotere)-Carboplatin	1	0.8
	Docetaxel (Taxotere)-Herceptin	1	0.8
	Docetaxel (Taxotere)-Liposomal Doxorubicin (Doxil)	1	0.8
	Docetaxel (Taxotere)-carboplatin-Herceptin	1	0.8
	Dose-dense AC followed by Paclitaxel (Taxol)	54	42.2
	Dose-dense AC followed by Paclitaxel (Taxol)-Albumin-bound Paclitaxel (Abraxane)	3	2.3
	Dose-dense AC followed by Paclitaxel (Taxol)-BlindedDrugTrial	3	2.3
	Dose-dense AC followed by Paclitaxel (Taxol)-Cytoxan-Taxotere	1	0.8
	Dose-dense AC followed by Paclitaxel (Taxol)-Herceptin	10	7.8
	Dose-dense AC followed by Paclitaxel (Taxol)-Herceptin-Taxotere	1	0.8
	Dose-dense AC followed by Paclitaxel (Taxol)-Zoladex	1	0.8
	Doxorubicin(Adriamycin)/Docetaxel(Taxotere)	1	0.8
	Doxorubicin(Adriamycin)/Docetaxel(Taxotere)-Taxol	1	0.8
	Doxorubicin(Adriamycin)/Paclitaxel(Taxol)-Herceptin	1	0.8
	Other GnRH agonist	1	0.8
	Paclitaxel (Taxol)-Herceptin	1	0.8
	TAC	1	0.8
	Trastuzumab (herceptin, anti-HER2mab)	2	1.6
SBR	1	41	20.5
	2	60	30
	3	99	49.5

2.D.3. Association between WAVE3 expression levels and cohorts paramaters

Variable	SBR1/ER+	SBR3/ER-	*p* value*
	Mean (SD)	Mean (SD)	
Age at Diagnosis (years)	51.33 (± 12.15)	54.86 (± 14.8)	0.172
Tumor Size (cm)	1.36 (± 1.07)	2.27 (± 1.99)	0.002
	n (%)	n (%)	
Lymph Node Status**			0.736
Negative	25 (62.5)	43 (57.3)	
Positive	15 (± 37.5)	32 (42.7)	
TNM Stage			0.009
Stage I	31 (81.6)	37 (50)	
Stage II	7 (18.4)	29 (39.2)	
Stage III	0 (0)	5 (6.8)	
Stage IV	0 (0)	3 (4.1)	
Her2 Status			0.01
Negative	39 (97.5)	58 (77.3)	
Positive	1 (2.5)	17 (22.7)	
Histology			0.144
Ductal	24 (60)	57 (75)	
Invasive	16 (40)	19 (25)	
Grade			<0.00001
I (well diff.)	7 (18.9)	19 (25)	
II (mod. diff.)	14 (37.8)	2 (2.7)	
III (poorly diff.)	16 (43.2)	71 (97.3)	

Figure 1. WAVE3 expression levels in CTCs are associated with ER-negative BC

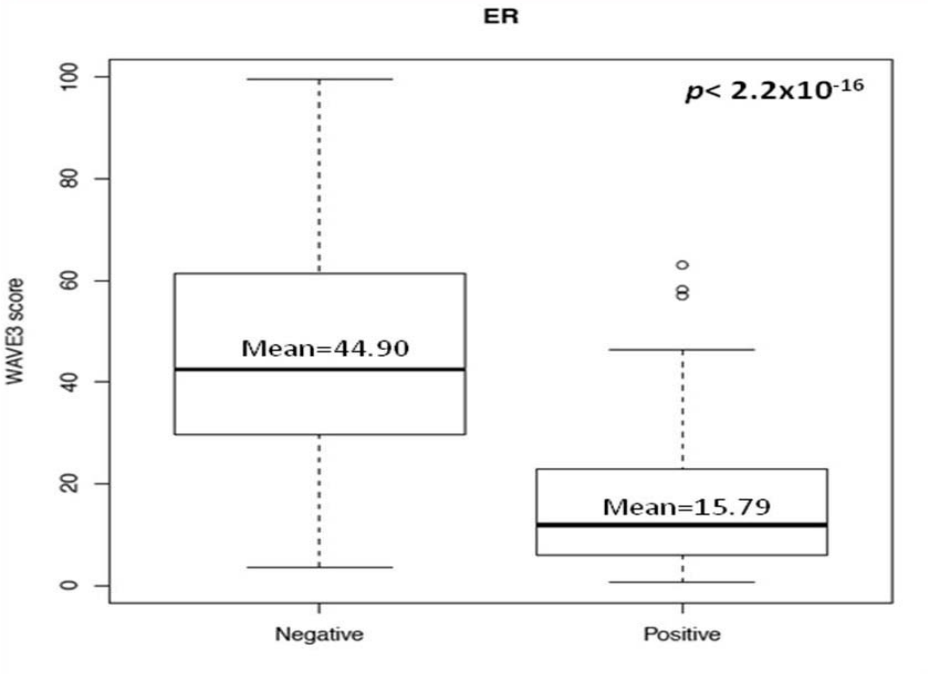


Figure 2. WAVE3 expression levels in CTCs are associated with PR-negative BC

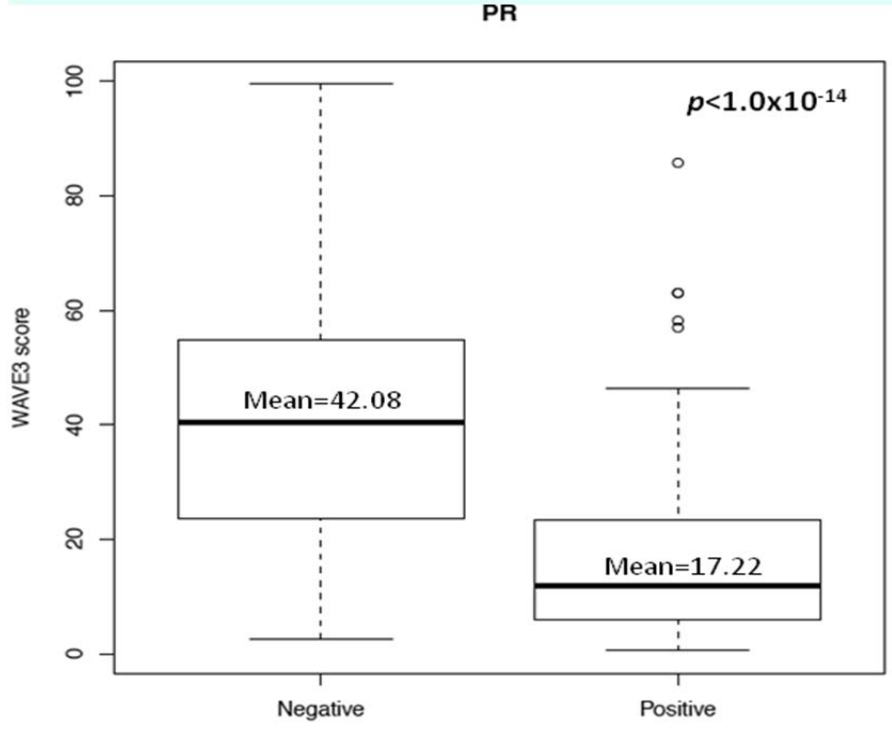


Figure 3. WAVE3 expression levels in CTCs are associated with tumor size in BC

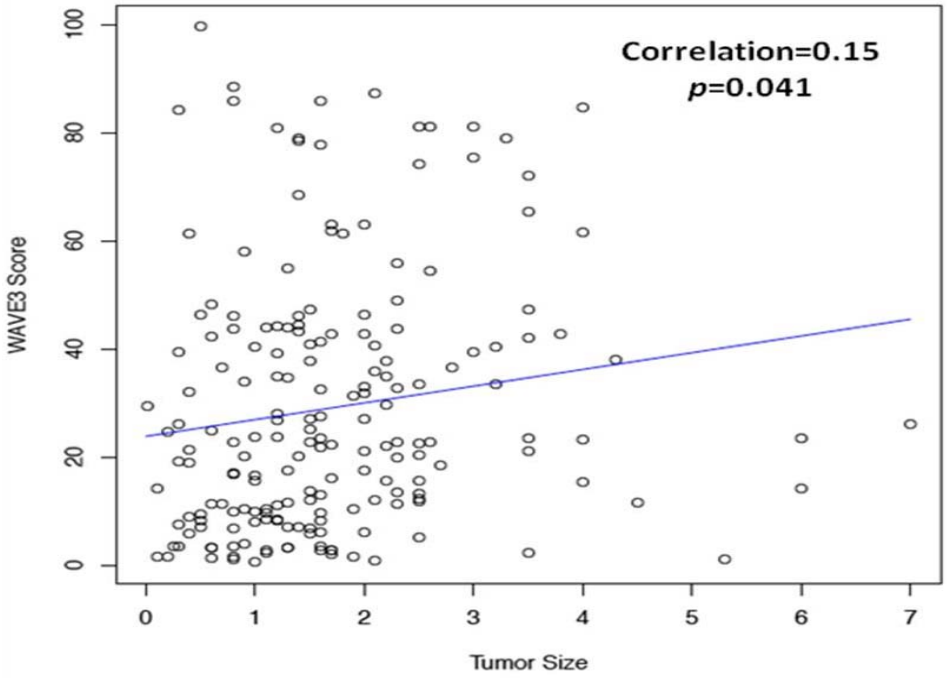
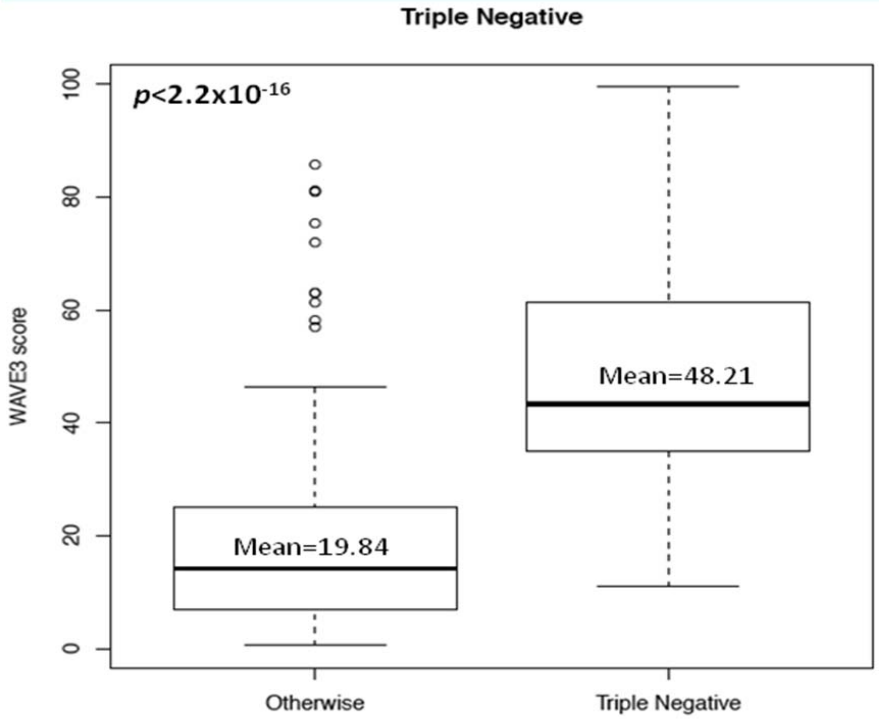


Figure 4. WAVE3 expression levels in CTCs are associated with triple-negative BC



The major finding from Aim2 is that WAVE3 expression levels in the CTCs are significantly associated with the triple-negative tumors and may be used as a marker for this BC subtype.

KEY RESEARCH ACCOMPLISHMENTS

Major conclusions from this study are:

Specific Aim 1: To test whether WAVE3 levels can be used as a predictive marker for the progression and metastasis of breast cancer.

The major findings from the WAVE3 staining in the primary breast cancer tumors are as follows:

1. WAVE3 staining correlates with tumor grade.
   We found a significant correlation between the tumor grade and WAVE3 staining levels. Low grade tumors show low WAVE3 score versus the high grade tumors which showed high WAVE3 score.

2. Low levels of WAVE3 staining correlate with reduced Distant Recurrence or Breast Cancer Related Mortality.
   We found a very significant correlation between the levels of WAVE3 staining in the primary tumors and the overall disease free survival. Those patients with low levels of WAVE3 staining in the primary tumors were found to live longer disease free after the primary tumors was removed compared to those patients with high levels of WAVE3 staining.

3. **The recurrence free survival is tumor grade-dependent.**
   We also found that a very significant correlation between the tumor grade and the overall disease free survival. Patients with grade 1 tumors and low WAVE3 staining tend to live longer with no detectable disease compared to those patients with grade 3 tumors and high levels of WAVE3 staining.

Specific Aim 2: To evaluate the prognostic value of WAVE3 expression levels in circulating tumor cells after the administration of adjuvant chemotherapy in women with operable breast cancer.

The major finding from the evaluation of WAVE3 expression levels in the blood of breast cancer patients is as follows.

4. **WAVE3 expression levels in the CTCs are significantly associated with the triple-negative tumors and may be used as a marker for this BC subtype.**

REPORTABLE OUTCOMES

The results from these studies have been presented at two major cancer research scientific meetings:

1. Rivera L, Khoury T, Tian L, Groman A, Watroba N, **Sossey-Alaoui K**, Kulkarni S. **WAVE3 over-expression is associated with adverse tumor characteristics and mortality in breast cancer.** Proceedings of 33rd CTRC-AACR San Antonio Breast Cancer Symposium. Abstract #P4-09-16, 2010.

Background: WAVE3 regulates actin polymerization and subsequent cell migration leading to enhanced metastatic potential. Based on pilot data that suggested WAVE3 expression was associated with high histologic grade and absence of estrogen receptor (ER) expression we hypothesized that WAVE3 expression would correlate with ER status and tumor grade in a matched group of breast cancer (BC) patients. WAVE3 expression was also analyzed in relation to adverse tumor characteristics, distant recurrence (DR) and BC specific mortality. Methods: Our institutional BC database was reviewed for patients who presented with, invasive BC from 1999-2009. Matching by stage and treatment was achieved for 61 patients with Scarff-Bloom-Richardson (SBR) grade 1 and ER+ tumors (SBR1/ER+) to 61 patients with SBR grade 3 and ER- tumors (SBR3/ER-). Cytosolic WAVE3 tumor expression was determined by immunohistochemistry. The product of stain intensity (0-3) and percentage of cells staining (0-100) was used to derive a WAVE3 score (0-300). The log rank test was utilized to compare BC specific mortality or distant recurrence free survival at various WAVE3 scores. A score of ≥212 was found to have the strongest association with poor outcome. The association between WAVE3 score and clinicopatholigic features, DR and BC specific mortality was assessed. Results: Increased frequency of Her2-neu (+) status, DR and BC specific mortality was noted in the SBR3/ER- group but WAVE3 score was no different between the two groups(Table1). In all 122 patients median WAVE3 score increased with tumor size (0.234, p=0.009), (+) lymph node status 200 vs. (-), 145, p =0.03, and stage (I, 160 vs. II,180 vs III, 240, p=0.012). There was no association between WAVE3 score and Her2-neu status(+200 vs. -180, p=0.51). In the SBR1/ER+ group only (+) lymph node status remained associated with WAVE3 score(+) 200 vs. (-) 130, p=0.02). In the SBR3/ER- group only lymph node status lost association with WAVE3 score (+)180 vs.(-)170, (p=0.50). DR and BC specific survival could only be assessed in the SBR3/ER- group. Median WAVE3 score was elevated with DR (240 vs. none, 160, p=0.03) and BC specific mortality (270 vs. none 170, p=0.004). A WAVE3 score ≥ 212 was associated with distant recurrence and BC specific mortality on Kaplan Meier analysis(p=0.01) and p<0.001) . On multivariate analysis a WAVE3 score ≥ 212 was associated with an increased risk for BC specific mortality (p=0.009). The association of DR and WAVE3 score ≥ 212 approached significance (p=0.068).

	SBR1/ER+	SBR3/ER-	p
Her2-neu +	5%(3/59)	33%(20/61)	<0.001
Median WAVE3 score	160	180	0.70
Distant recurrence	0	16%(10/61)	0.001
BC specific mortality	0	11% (7/61)	0.006

Conclusion: WAVE3 expression is not associated with tumor grade, and ER or Her2 neu status. WAVE3 is associated with tumor size, stage, DR and BC specific mortality in the high risk SBR3/ER- group. A WAVE3 score of ≥ 212 is associated with distant recurrence and breast cancer specific mortality on univariate analysis and BC specific mortality on uni- and multivariate analysis. WAVE3 expression may contribute to adverse outcome in high risk breast cancer patients.

2. Augoff K, Zhang L, Rivera L, Khoury T, Tian L, Groman A, Watroba N, Plow EF, Kulkarni S, **Sossey-Alaoui K.** Increased expression levels of WAVE3 are associated with breast cancer progression and metastasis. Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research. Abstract #.3206, 2011.

Cancer metastasis is a complex process, usually requiring cancer cells to escape from the primary site, survive in the blood/lymph system and then to colonize a distant site. We have shown that the expression levels of WAVE3 are significantly correlated with advanced stages of breast cancer (BC). Our published studies have now established a function for WAVE3 as a metastasis promoter gene in BC. We, therefore, used immunohistochemistry to test the prognostic value of WAVE3 in the progression and metastasis of BC. We analyzed the expression levels of WAVE3 in 61 patients with Scarff-Bloom-Richardson (SBR) grade 1 and ER+ tumors (SBR1/ER+), which were matched to 61 patients with SBR grade 3 and ER- tumors (SBR3/ER-). The product of staining intensity (0-3) and percentage of stained cells (0-100) was used to derive a WAVE3 score (0-300). In all 122 patients, median WAVE3 score increased with tumor size ($p=0.009$), (+) lymph node status ($p=0.03$), and stage ($p=0.012$). Distant recurrence (DR) and BC specific survival could only be assessed in the SBR3/ER- group, where median WAVE3 score was elevated with DR and BC specific mortality. A WAVE3 score ≥ 212 was associated with distant recurrence and BC specific mortality in Kaplan Meier analysis ($p=0.03$), as well as with an increased risk for BC specific mortality ($p=0.004$). From the immunohistochemistry analysis we concluded that WAVE3 is associated with tumor size, stage, DR and BC specific mortality in the high risk SBR3/ER- group. WAVE3 expression may, therefore, contribute to adverse outcome in high risk breast cancer patients.

On the other hand, WAVE3 is expressed at very low levels in the hematopoietic cells, but its expression levels are increased in the circulating tumor cells (CTC) as a result of early tumor cell dissemination even in patients with small tumors. We used this WAVE3-specific characteristic to evaluate the prognostic value of WAVE3 expression levels in CTCs in women with operable breast cancer. We used quantitative real-time RT-PCR to assess the expression levels of WAVE3 in the blood of 200 breast cancer patients, representative of different stages and pathology of the disease, after they have completed surgery and chemotherapy. The expression data was correlated with the clinicopathiological parameters One of the major findings was that the WAVE3 expression levels was significantly higher in the triple negative breast cancer ($p<10^{-18}$).

This study is aimed to define the role of WAVE3 in the progression and metastasis of breast cancer and to provide a fundamental and sound scientific basis upon which the role of WAVE3 can be evaluated in breast cancer and utilized as a prognostic indicator in the clinic setting, specifically predicting women at increased risk of distant metastasis.

CONCLUSION.

The major conclusions from this study so far are:

- ❖ WAVE3 score is positively correlated with tumor size, lymph node status and pathologic stage.
- ❖ Only patients in the SBR3/ER- cohort experienced distant recurrence or disease specific mortality. WAVE3 scores were higher in patients with adverse clinical outcome.
- ❖ A WAVE3 score≥ 212 was associated with breast cancer specific survival on uni - and multivariate analysis.
- ❖ WAVE3 score is independently associated with an increased risk for breast cancer specific mortality
- ❖ WAVE3 score may be able to predict adverse outcome in high risk breast cancer patients.
- ❖ WAVE3 expression levels in the CTCs are significantly associated with the triple-negative tumors and may be used as a marker for this BC subtype.

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Appendices manuscript submitted to PLos One

PLoS ONE

Increased Expression Levels of WAVE3 are Associated with the Progression and Metastasis of TNBCs

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Increased Expression Levels of WAVE3 are Associated with the Progression and Metastasis of TNBCs
Short Title:	WAVE3 promotes metastasis of TNBC
Corresponding Author:	khalid sossey-alaoui, PhD Cleveland Clinic Lerner Research Institute Cleveland, OH UNITED STATES
Keywords:	WAVE3, TNBC, Breast cancer, Metastasis, Invasion-metastsis-cascade, Disease-specific Mortality, Epthelial-Mesenchymal Transition
Abstract:	Background: Breast Cancer (BC) is a heterogeneous disease comprised of at least five genetically distinct subtypes, which together form the second leading cause of cancer death in women in the United States. Within BC subtypes, those classified as Triple Negative BCs (TNBCs) exhibit dismal survival rates due to their propensity to develop distant metastasis. We have identified the WAVE3 protein, which is a critical regulator of actin cytoskeleton dynamics that are required for the motility and invasion of cancer cells through its activation of Arp2/3 complex, as a key regulator of the different steps of the invasion-metastasis cascade in BC, especially in the more aggressive TNBCs. Our published studies have also shown that elevated expression levels of WAVE3 in the TNBC directly contribute to their increased invasion and metastasis potentials both in vitro and in vivo in murine models of BC metastasis. Methodology/Principal Findings: Herein, we utilized both immuno-histo-chemistry (IHC) of primary human BC tumors as well as quantitative real-time RT-PCR of WEVE3 in the peripheral blood of BC patients to clearly establish that WAVE3 is a predictive marker of overall BC patient survival. High levels of WAVE3 were predictive for reduced distant recurrence-free survival as well as for decreased disease-specific mortality. Our analysis of WAVE3 expression levels the peripheral blood of BC patients have shown that WAVE3 is highly expressed in the blood of patients with who developed metastatic breast cancer compared to those who did not. WAVE3 expression was also highly upregulated in the blood of BC patients with the more aggressive TNBC subtype. Conclusions: Together, these findings establish WAVE3 as a novel marker for increased risk of breast cancer specific mortality and for the metastatic potential of the TNBCs, and also identify WAVE3 as an attractive therapeutic target for the treatment of metastatic BC.
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To: whom it may concern
PLoS One Editorial Team

Cleveland, May 08, 2012

Re: Submission of original manuscript

Dear Members of the Editorial Team

Enclosed please find the submission of our manuscript entitled: **"Increased Expression Levels of WAVE3 are Associated with the Progression and Metastasis of TNBCs**" Kulkarni et al., for consideration for publication in **PLoS ONE**.

Our manuscript reports the significant finding that WAVE3 is a predictive marker of overall breast cancer (BC) patient survival. Our published data, using both in vitro and in vivo pre-preclinical mouse model for BC metastasis, clearly identified WAVE3 a BC metastasis promoter gene. The correlation between WAVE3 expression levels and disease outcome has, however, not been extensively investigated. In the present study, we utilized immuno-histo-chemistry on primary human BC tumors to clearly demonstrate that high levels of WAVE3 are predictive for reduced distant recurrence-free survival as well as for decreased disease-specific mortality in BC patients. Using a second independent BC patient cohort, our analysis of WAVE3 expression levels the peripheral blood patients showed that WAVE3 is highly expressed in the blood of patients with who developed metastatic breast cancer compared to those who did not. WAVE3 expression was also highly upregulated in the blood of BC patients with the more aggressive TNBC subtype.

Together, our findings establish WAVE3 as a novel marker for increased risk of BC-specific mortality and for the metastatic potential of the TNBCs, and also identify WAVE3 as a significant therapeutic target for the treatment of metastatic BC.

The authors do not have any conflict of interest to declare and affirm that the manuscript has not been published previously and is not being considered concurrently by another journal.

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Increased Expression Levels of WAVE3 are Associated with the Progression and Metastasis of TNBCs

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Running Title: WAVE3 promotes metastasis of TNBC

ABSTRACT

Background: Breast Cancer (BC) is a heterogeneous disease comprised of at least five genetically distinct subtypes, which together form the second leading cause of cancer death in women in the United States. Within BC subtypes, those classified as Triple Negative BCs (TNBCs) exhibit dismal survival rates due to their propensity to develop distant metastasis. We have identified the WAVE3 protein, which is a critical regulator of actin cytoskeleton dynamics that are required for the motility and invasion of cancer cells through its activation of Arp2/3 complex, as a key regulator of the different steps of the invasion-metastasis cascade in BC, especially in the more aggressive TNBCs. Our published studies have also shown that elevated expression levels of WAVE3 in the TNBC directly contribute to their increased invasion and metastasis potentials both in vitro and in vivo in murine models of BC metastasis. **Methodology/Principal Findings:** Herein, we utilized both immuno-histo-chemistry (IHC) of primary human BC tumors as well as quantitative real-time RT-PCR of WEVE3 in the peripheral blood of BC patients to clearly establish that WAVE3 is a predictive marker of overall BC patient survival. High levels of WAVE3 were predictive for reduced distant recurrence-free survival as well as for decreased disease-specific mortality. Our analysis of WAVE3 expression levels the peripheral blood of BC patients have shown that WAVE3 is highly expressed in the blood of patients with who developed metastatic breast cancer compared to those who did not. WAVE3 expression was also highly upregulated in the blood of BC patients with the more aggressive TNBC subtype.

Conclusions: Together, these findings establish WAVE3 as a novel marker for increased risk of breast cancer specific mortality and for the metastatic potential of the TNBCs, and also identify WAVE3 as an attractive therapeutic target for the treatment of metastatic BC.

INTRODUCTION

Breast cancer is the most common malignancy diagnosed in women and the second leading cause of cancer mortality after lung cancer [1–4]. Metastasis is responsible for ~90% of deaths in patients with solid tumors [5–10], including those originating in the breast [11–13]. The risk of developing distant metastasis and therefore prognosis in BC is associated with the presence of a number of pathologic characteristics: positive lymph node status, increasing tumor size and histologic grade. BC is a heterogeneous disease comprised of at least five genetically distinct subtypes. For instance, luminal BCs, which also tend to be estrogen receptor positive (ER+) and low grade, have the lowest risk of developing distant metastases and have the best prognosis. In the other end of the spectrum, the basal BC subtypes, which also include the Triple Negative BCs (TNBCs) exhibit dismal survival rates due to their highly aggressive and metastatic behavior, and to their propensity to rapidly recur [14–20]. Genetically, TNBCs are characterized by lack of expression of hormone receptors (ER-α and PR) and HER2, harbor BRCA1-defects and/or deficiencies, and may remain p53-positive [21], which makes them refractory to hormonal therapy, further contributing to the risk of aggressive relapse and dismal survival rates amongst women bearing TNBCs [6,9,10,22,23].

Cancer metastasis is a complex and multistep process, requiring cancer cells to escape from their primary site, survive in the blood/lymph system and then to establish a new niche at a distant site. This complex process also involves cell motility, epithelial mesenchymal transition (EMT) and the multiple steps of the invasion-metastasis cascade of cancer cells [5,10]. We have shown that the WAVE3 protein, which is a critical regulator of actin cytoskeleton dynamics through its activation of Arp2/3, is required for the motility and invasion of cancer cells [24–27]. Specifically, our published studies have demonstrated that WAVE3 expression controls cell

shape and is required for lamellipodia formation, which in turn is tightly linked to the distinctive migratory and invasive phenotypes of tumor cells [25,28]. Mechanistically, we have shown that loss of WAVE3 expression results in the down-regulation of metalloproteinases that control invasive properties [24]. We have also shown that WAVE3 is expressed at high levels in both human breast cancer cell lines and tumors [27]. Most importantly, we found that stable knockdown of WAVE3 prevents metastasis of the TNBC MDA-MB-231 cells in a mouse model [27], supporting the function of WAVE3 as a metastasis promoter gene.

Given the clinical characteristics of high-grade breast cancers, we hypothesized that WAVE3 might be expressed in higher levels compared to low grade tumors and this elevated expression might contribute to the increased metastatic potential seen in the high-grade tumors compared to low-grade tumors. To answer this question, we conducted two retrospective studies using two different BC patients' cohorts, in an attempt to isolate the effect of the levels of expression of WAVE3 on BC progression and metastasis. In the first study we identified patients from two very different groups of patients (ER(+)/modified Scarff-Bloom-Richardson (mSBR1) and ER(-)/mSBR3) in our breast cancer database and tumor bank and assessed for WAVE3 expression levels in the primary tumors using IHC. Correlation of WAVE3 expression levels to the patients' clinicopatholological characteristics and the disease outcome and led to the following findings: *(i)* WAVE3 is highly expressed in malignant vs. adjacent normal ductal epithelium, *(ii)* WAVE3 expression is positively correlated with adverse clinicopathologic parameters, *(iii)* WAVE3 expression is increased in the tumors of patients who developed distant metastases, *(iv)* WAVE3 expression levels are positively correlated with reduced distant recurrence free survival and with decreased disease specific survival and *(v)* we concluded that WAVE3 is an independent marker

for increased risk for breast cancer specific mortality as well as for decreased distant-recurrence-free survival.

In the second study we evaluated the prognostic value of WAVE3 mRNA expression levels in the circulating tumor cells in the peripheral blood of women with operable breast cancer, based on the unique characteristic of the lack of WAVE3 expression in the peripheral blood mononuclear cells (PBMCs). Analysis of WAVE3 expression levels in the blood of 200 BC patients and correlation with the patients' clinical data revealed that (*i*) WAVE3 mRNA is highly expressed in the peripheral blood of patients with metastatic breast cancer, and (*ii*) WAVE3 expression levels in the blood of BC patients correlates positively with the aggressive TNBC subtype. We therefore concluded that, together, our data clearly identify WAVE3 as a novel biomarker for the progression and metastasis of breast cancer. Our data also support the use of WAVE3 a specific marker for the most aggressive forms of BC, i.e., the TNBC.

MATERIALS AND METHODS

Materials. The antibodies used in this study were: rabbit anti-WAVE3 from New England Peptide, Inc.; goat HRP-conjugated anti-mouse IgG and goat HRP-conjugated anti-rabbit IgG from Calbiochem. We used the On-Targetplus SMARTpool (Thermo Scientific) siRNA L-1012301-00-0010 to knockdown the expression of WAVE3.

Patient Selection:

Ethics Statement: This study was conducted after approval from the institutional review board (IRB) at Roswell Park Cancer Institute (RPCI), who also waived the need for consent. Consent was not needed for this study since there was no interaction with patients, which were enrolled, based on their existing, available medical information, and that information was used in a de-identified fashion.

The RPCI Breast Surgery Database was reviewed to identify patients who received standard surgical therapy with documented negative tumor margins for non-metastatic, invasive breast cancer from 1999 to 2009. Patients who had tumors with an ER(+)/mSBR grade I were matched to patients who had ER(-)/mSBR grade III tumors. These two groups were also matched by pathologic tumor stage, type of surgical treatment, and whether adjuvant therapy was given. Other data including age at diagnosis, tumor size, number of positive lymph nodes, total number of lymph nodes retrieved, ER and PR Allred score, Her-2 neu receptor status, type of systemic therapy, radiation therapy, recurrence, and mortality were also collected. Recurrence free survival was defined as the time from diagnosis to ipsilateral chest wall or breast recurrence, for local recurrence, or as time from diagnosis to first recurrence in a distant site, for distant recurrence. Disease specific survival was defined as time from diagnosis to breast cancer related mortality or last follow up.

For the investigation of the expression levels of WAVE3 in the peripheral blood of breast cancer patients, blood samples were obtained from 200 patients with operable breast cancer from different stages before the surgical resection of the primary tumor and before the initiation of any kind of treatment. The control population consisted of blood samples from 200 healthy females without cancer history. In addition blood samples were obtained from ten patients with metastatic breast cancer. These de-identified samples all came from the RPCI Databank and Biorepository.

WAVE3 Immunohistochemistry. Paraffin sections were cut at 5 μm, placed on charged slides, and dried in a 60°C oven for 1 hour. Room temperature slides were deparaffinized in three changes of xylene and rehydrated using graded alcohols. Endogenous peroxidase was quenched with aqueous 3% H_2O_2 for 10 minutes and washed with phosphate buffered saline/Tween® 20 (PBS/T). Antigen retrieval was then performed with citrate buffer, pH 6, in a microwave for 10 minutes and allowed to cool for 15 minutes followed by a PBS/T wash. The slides were then placed on the DAKO autostainer, and the following program was run: PBS/T wash followed by a 30-minute incubation in 0.03% casein in PBS/T, and a 1-hour incubation at room temperature with 0.5 μg/ml rabbit anti-WAVE3 [27]. Rabbit IgG at 0.5 μg/ml was used on a duplicate slide in place of the primary antibody as a negative control. This was followed by overnight incubation with a horseradish peroxidase (HRP) labeled polymer conjugated to a secondary anti-rabbit antibody (DAKO EnVision™+ System, HRP, code k4010). Slides were washed in PBS/T and staining was completed by a 5 minute incubation with 3,3'-diaminobenzidine (DAB)+ substrate-chromogen (DAKO) which resulted in a brown-colored precipitate at the antigen site. The slides were then counterstained with hematoxylin, dehydrated, cleared, and coverslipped.

Histopathological Analysis. Tumor specimens were reviewed and scored by a single pathologist (T.K.) mSBR grade was verified for each specimen [29–31] and ER status was quantified using the Allred scoring system if not previously recorded [32]. Cytosolic WAVE3 expression was detected utilizing IHC staining as noted above. WAVE3 expression was scored as follows: 0 for negative, 1 for weak, 2 for moderate, and 3 for strong. The percentage of positively stained cells in each scored field was recorded. The product of the intensity score and percentage of positively stained cells is the WAVE3 score (WAVE3 score = intensity X % of positively stained cells) [27]. WAVE3 expression was reported both for tumor and adjacent benign ductal epithelium. Adjacent benign epithelium was assessed to determine if there was any difference in the level, pattern, or intensity of WAVE3 expression between tumor and normal ductal epithelium.

Preparation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) are isolated the red blood cells are lysed by mixing 2 ml of whole blood with 6 ml of RBC for 10 minutes at room temperature, followed by centrifiguation for 1 min at 15000 g. The peletted PBMCs are then used for total RNA isolation by using Trizol LS reagent (Gibco, Life Sciences, BRL, Grand Island, NY) according to the manufacturer's instructions. All RNA preparation and handling steps are performed in a laminar flow hood, under ribonuclease-free conditions. The isolated RNA is dissolved in diethylpyrocarbonate-treated water and stored at –80°C until used. RNA integrity is routinely tested by PCR amplification of the ß-actin housekeeping gene. RNA extracted from WAVE3-overexpressing breast cancer cell line MDA-MB-231 and EVB-Lin lymphoblastoid cell line are used as positive and negative controls, respectively.

Semi-quantitative and Real-time quantitative–PCR. Total RNA was extracted from established cancer cell lines using TRIzol reagent (Invitrogen), following to the manufacturer's instructions. cDNA was generated and used as a template for semi-quantitative RT-PCR performed as previously described [24,25,27,33]. Real-time quantitative RT-PCR was performed using the respective gene-specific primers (SABiosciences) and the RT^2 SYBR Green/Fluorescein qPCR Master Mix (SABiosciences) following the manufacturer's instructions. Quantitative PCR was performed on the BioRad iCycler PCR system where the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The cycle threshold (Ct) values were calculated with SDS 1.4 software (Bio-Rad). The expression levels of each transcript were normalized using the $2^{-\Delta\Delta Ct}$ method [34,35] relative to GAPDH. The ΔCt was calculated by subtracting the Ct values of GAPDH from the Ct values of the transcript of interest. The ΔΔCt was then calculated by subtracting ΔCt of the matching normal human breast tissue from the ΔCt of cancer tissues, or the ΔCt of MCF10A cell line for the established cancer cell lines. Fold change in the gene was calculated according to the equation $2^{-\Delta\Delta Ct}$.

Matrigel Invasion Assay. The invasive potential of the parental and transfected cells was assessed using the Matrigel invasion chambers from BD Biosciences as described [24,27,33].

Statistical Analyses.

General Considerations: Categorical variables were described by frequency distributions and contingency tables, while symmetric and skewed continuous variables were summarized by mean, standard deviation (STD) and Tukey summaries (median, 25% and 75% quartile), respectively. Statistical analysis for comparing two groups with regard to continuous variables were performed utilizing t-test or the nonparametric alternative, the Mann-Whitney rank sum. When comparing more than two groups with continuous variables the Kruskal-Wallis One Way

Analysis of Variance on Ranks (ANOVA), non-parametric test with exact p values were performed. Comparison of groups with regard to categorical data was performed with the Chi square test using exact p values. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. The Bonferroni adjustment was implemented if there are any multiple testing issues, and significance will be assessed at the 0.05 level. Statistical analyses were performed using the statistical software R (http://www.r-project.org/) and using SigmaStat for Windows Version 3.5.0.54 (Systat Software, Inc. Chicago, IL, USA).

WAVE3 immunohistochemistry:

Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. To determine the relative contribution of the WAVE3 score to distant disease free recurrence and disease specific survival the WAVE3 score was dichotomized as positive or negative. The midpoint of the difference between the 75% quartile for the WAVE3 score of patients with no distant recurrence or breast cancer related mortality and the 25% quartile was used. The median and 25-75% quartile of the WAVE3 scores for all patients with distant tumor recurrence and breast cancer related mortality were determined by the rank sum test and compared to similar values in patients without distant recurrence or breast cancer related mortality. Covariates considered for multivariate analysis included tumor size, number of involved lymph nodes, Her2 neu status, receipt of neoadjuvant therapy and WAVE3 Score. The regression coefficient (beta), the hazard ratio and 95% confidence intervals were obtained for each covariate.

WAVE3 expression in the blood: First, to detect the association of expression levels with the ER status, race, histologic grade, TNM staging, size, lymph node status and tumor subtype, Chi-

square tests and a multivariate linear model were applied for univariate and multivariate analysis. For the correlations of WAVE3 expression levels between the different subtypes of BCs we used logistic models of tumor status and response outcome as predictors adjusted for age and race. Second, the association between expression levels and the time-to-event endpoints were analyzed using the Cox Proportional Hazards Model to estimate the effect of WAVE3 expression levels on recurrence and survival rates adjusted against clinicopathological factors potentially predictive of clinical outcome.

RESULTS

WAVE3 expression levels positively correlate with the aggressiveness of breast cancer cell lines. In our previously published studies [24,26,27,33] we showed that WAVE3 is highly expressed in the aggressive BC MDA-MB-231 cells, and that WAVE3 activity is required for the invasion of this cell line both *in vitro* as well as in *in vivo* mouse models for breast cancer tumor progression and metastasis [24,26,27,33]. On the other hand, WAVE3 expression was found to be very low in the non-invasive MCF7 BC cell line, and that over-expression of WAVE3 in MCF7 cells was sufficient for increasing the invasiveness potential of this otherwise non-invasive BC cell line [26]. More importantly, MDA-MB-231 and MCF7 cells belong to two distinct BC subtypes, i.e., triple-negative (TN) of basal subtype and luminal subtype, respectively. Based on these observations, we hypothesized that WAVE3 may be associated with the TNBC of basal subtype. We therefore sought to determine whether this property of WAVE3 can be extended to other BC cell lines of luminal versus TNBC of basal subtype. WAVE3 expression levels were assessed by western blotting in a series of six BC cell lines and was found to be highly expressed in MDA-MB-231, BT549 and MDA-MB-435s, all of TN basal subtype, while WAVE3 expression levels were comparatively very low in MCF7, T47D and SKBr3 BC cell lines of luminal subtype (Fig. 1A). Expression levels of WAVE3 in these cell lines also correlated with their invasiveness potential in the *in vitro* Matrigel invasion assay (Fig. 1B and 1C). The TNBC cell lines (MDA-MB-231, BT549, and MDA-MB-435s), which overexpress WAVE3 also exhibit high invasiveness potential, as opposed to the luminal BC cell lines (MCF7, T47D and SKBr3). Knockdown of WAVE3 expression in the TNBC cell lines using siRNA specific to WAVE3, resulted in a significant inhibition of their invasiveness potential (Figure 2), and this effect is specific to the loss of WAVE3 since the expression levels of other

WAVE isoforms were not affected by the loss of WAVE3 expression. Of note, neither the control siRNA nor the WAVE3 siRNA affected the viability or the proliferation of these cell lines (not shown).

WAVE3 is a biomarker for breast cancer progression and metastasis. Based on the findings from the in vitro analyses described above we concluded that WAVE3-mediated regulation of invasion of BC cell lines is not restricted to MDA-MB-231 cell line but can be generalized to several other TNBC cell lines of basal subtype. We therefore expanded our analyses in a retrospective study where we used annotated human specimens and sought to investigate whether our in vitro findings with established BC cell lines can be translated to human tumors.

Patient Population: Initially, 228 patients were identified from the RPCI Breast Surgery database based on their tumors ER status and histologic grade mSBR grading. Archived tumor specimens from the RPCI tissue repository were evaluated for specimen adequacy and ultimately, 128 cases with complete clinicicopoathologic data were available for analysis of WAVE3 expression by IHC. Sixty-four patients with tumors that were ER(+)/mSBR grade I and 64 patients that were ER(-)/mSBR grade III were identified. The WAVE3 score (median score) in the ER(+)/mSBR1 was 160 and the WAVE3 score in the ER(-)/mSBR3 was 180. Comparison of the clinical data revealed that the patients in each group were matched for age, tumor size, lymph node status, and adjuvant treatment (Table 1). The median follow up for both groups was 36 months. The ER(-)/mSBR3 group demonstrated a significantly increased prevalence of Her2neu receptor positivity (ER(-)/mSBR3, 31.2% vs. ER(+)/mSBR1, 4.7%, $p<0.001^{*}$). As expected, all recurrences (local and distant) were also significantly increased in the group ER(-)/mSBR3 26.6% vs. ER(+)/mSBR1, 1.6%, $p<0.001^{*}$. Distant recurrences accounted for the majority of recurrences and were seen in 17.2% of the ER(-)/mSBR3 group with no distant

recurrences occurring in the mSBR1 group ($p<0.001^{*}$). Breast cancer specific mortality in the ER(-)/mSBR3 group was 12.5% vs. 0% in the ER(+)/mSBR1 group, p=0.003 during the follow-up period. Analysis of recurrence free survival revealed a statistically significant decrease in local recurrence free survival in the mSBR3 group, p<0.001. Similar results were demonstrated for analysis of distant recurrence free survival and disease specific survival, p=0.023 and 0.005 respectively.

WAVE3 is highly Expressed in Malignant vs. Adjacent Normal Ductal Epithelium. We used a WAVE3-specific antibody to perform immunohistochemistry on the specimens described above to evaluate the levels WAVE3 expression [27]. First we evaluated WAVE3 expression levels in the tumor epithelial cells vs. the adjacent normal ductal epithelial cells (Table 2). As expected, we found that the median WAVE3 score was significantly higher in the tumor cells compared the adjacent normal ductal epithelium (Tumor WAVE3, 180 vs. Benign WAVE3, 65, p<0.001). Of note, no expression of WAVE3 protein was seen in the surrounding stromal tissue. These data clearly confirm our preliminary findings where we used a much smaller cohort to first report increased levels of WAVE3 in human breast cancer tumors [27]. Because differences in expression in the surrounding epithelial tissue may also be involved in a tumor's metastatic potential, the possibility that the absolute difference in WAVE3 expression between normal and malignant epithelial cells was also assessed in the two groups, ER(-)/mSBR3 and ER(+)/mSBR1. This differential WAVE3 score demonstrated no difference between the two groups (Table 2). A representative staining of WAVE3 in one mSBR1 and one mSBR3 tumor is shown in figure 3 where a clear difference in staining can be seen between the ER(+)/mSBR1 (low score, Fig. 3A&B) and ER(-)/mSBR3 (high score, Fig. 3C&D).

WAVE3 expression is positively correlated with adverse clinicopathologic parameters. Next we combined the two groups and assessed the relationship between the WAVE3 score in the tumors and their associated clinicopathologic features. Tumor WAVE3 score was significantly increased in patients with lymph node metastases compared to those without lymph node metastases (lymph node positive, 200 vs. lymph node negative, 140, p=0.017, Table 3). Pearson product moment correlation also demonstrated a significant and direct relationship between the number of involved lymph nodes and WAVE3 score (correlation coefficient=0.224, p=0.0121). A similar analysis revealed a statistically significant, direct association between WAVE3 score and tumor size (correlation coefficient=0.226, and p=0.0102). Furthermore, as would be expected, there was a statistically significant increase in tumor WAVE3 score with increasing pathologic stage, which is comprised of tumor size and lymph node status (p=0.023, Table 3). No significant difference was, however, found between the WAVE3 score and the Her2neu status (Table 3). The overall data, however, established a clear positive correlation between WAVE3 expression levels in the primary tumors and severe/adverse disease characteristics, and, led us, therefore to speculate that increased WAVE3 expression levels in the primary tumors may be part the main driving force behind the progression of breast cancer to more aggressive stages.

To further support this hypothesis, we analyzed the expression levels of WAVE3 against two of the major disease outcomes, i.e., distant recurrence-free survival and disease-specific survival.

WAVE3 expression is increased in the tumors of patients who developed distant recurrence. In the combined data set, although, the median WAVE3 score was $1^{1/2}$-fold higher in those patients who developed distant recurrence compared to those who had no recurrence (distant recurrence 220, *vs.* no recurrence, 160, Table 4), this difference did not, however, achieve statistical

significance, which could be attributed to the inclusion in the analyses of patients who developed local recurrence (median WAVE3 score, 70). Local recurrence can often be the result of occult residual disease rather than tumor biology. Furthermore, when assessed by survival status, the patients who suffered a breast cancer related mortality had a statistically significantly higher WAVE3 score than those surviving (breast cancer related mortality, 255 vs. surviving, 160, p=0.028, (Table 4).

WAVE3 expression levels are positively correlated with reduced overall survival, reduced distant recurrence free survival and with decreased disease specific survival. To assess the association of WAVE3 score with distant recurrence and survival outcomes a threshold value needed to be established to create a dichotomous value. In the absence of a known value, the midpoint between the 75% quartile for the no distant recurrence group (100) and the 25% quartile for distant recurrence group (240) was used as the threshold to dichotomize the WAVE3 score as positive or negative. The value obtained was 212. Using this value in the univariate Kaplan-Meier modeling analysis, we found that a WAVE3 score >212 was significantly associated with reduced overall survival (p=0.0093), with reduced distant recurrence free survival (p=0.0409), and with decreased disease specific survival on (p=0.0005, Figure 4 and Supplemental Table S1). Lymph node status and tumor size were also predictive of disease and survival outcomes in the univariate analysis (Supplemental Table S1). On multivariate analysis, using a multivariable Cox proportional hazards modeling, a tumor WAVE3 score >212 was also significantly predictive of reduced overall survival (p=0.027, Supplemental Table S2), reduced distant recurrence free survival (p=0.027, Table 5 and Supplemental Table 2) and reduced disease-specific mortality (p=0.01, Table 6 and Supplemental Table S2).

Together, the data derived from the immunohistochemistry analysis of WAVE3 expression levels in 128 breast cancer specimens and its correlation with the tumor-associated clinical and pathological parameters as well the disease and survival outcomes, clearly demonstrated a significant association between WAVE3 expression levels and BC progression and metastasis. Our data also identified WAVE3 score is an independent marker for increased risk of BC specific mortality as well as for decreased distant-recurrence-free survival.

Evaluation of the prognostic value of WAVE3 expression levels in circulating tumor cells in the peripheral blood of women with operable breast cancer. In normal physiological conditions WAVE3 is expressed at very low in the blood cells [36,37]. On the other hand, we found that WAVE3 mRNA levels were much higher in the blood of metastatic breast cancer patients compared to their disease-free counterparts (see below). Distant metastases are the results of the proliferation in the new site of circulating tumor cells (CTCs) that have originally escaped the primary tumors and disseminated in circulation. Based on this widely accepted assumption and on our observation that WAVE3 is expressed at very low levels in the blood cells, we hypothesized that increased WAVE3 levels in the blood of metastatic BC patients might be a direct result of the presence of CTCs in the circulating blood of these metastatic BC patients. We therefore decided to conduct second clinical study whereby we sought to evaluate the prognostic value of WAVE3 expression levels in the peripheral blood which may also contain circulating tumor cells, in women with operable breast cancer from different stages.

WAVE3 mRNA is highly expressed in the peripheral blood of patients with metastatic breast cancer. We have shown that WAVE3 is highly expressed in the metastatic BC cell lines (Fig. 5A). We and others have also reported that WAVE3 is expressed at very low levels in the peripheral blood mononuclear cells (PBMC) [37,38]. We performed a pilot study, which also

served as a proof of principle experiment, to determine that our RT-PCR conditions will allow for the detection of WAVE3 mRNA in samples where MDA-MB-231 cells were mixed in serial decreasing numbers with the EVB-Lin lymphoblastoid cells. Indeed, we were able to detect WAVE3 transcripts in RNA samples where MDA-MB-231 and EVB-Lin cells mixed in a ratio as low as $1:10^6$ (Figure 5A), and by doing so we further established the sensitivity of our assay. Next we used both semi-quantitative RT-PCR (Figure 5B) and quantitative real-time-RT-PCR (Fig. 5C) to monitor the expression levels of WAVE3 in PBMC collected from ten patients with metastatic BC and ten healthy female controls with no known cancer history. WAVE3 expression levels were found to be significantly higher in the metastatic BC patients compared to their control counterparts, suggesting the presence of a significant number of CTCs the blood of these patients that ultimately resulted in the development of the metastatic disease. In one case (BC8) WAVE3 levels were more than 4000 times higher than the normal controls. This result also establishes a direct association between WAVE3 expression levels in the circulating blood of BC patients and the risk of development of the metastatic disease, which led us to the next step where we sought to evaluate the prognostic value of WAVE3 expression levels in circulating tumor cells in women with operable breast cancer who have not yet developed metastatic BC at the time when the blood samples were collected.

WAVE3 expression levels in the blood of BC patients correlate positively with the aggressive TNBC subtype. Patient population: 200 BC patients who underwent treatment at RPCI were identified from the archived BC blood database, and were evaluated for specimen adequacy and completeness of clinicicopoathologic information (Supplemental Table S3). WAVE3 expression levels were determined by quantitative real time RT-PCR and correlated with the patients clinicopathiological parameters.

The tumor nuclear grade is used as an initial assessment on how quickly the cancer may develop whereby grade 1 means low histologic grade and a favorable outcome, whereas grade 3 means high histologic grade and is often associated with a more unfavorable outcome. We found a significant positive correlation between the blood WAVE3 expression levels and the tumor nuclear grade (Figure 6A), with a mean of 38.48 in nuclear grade III tumors *versus* 10.54 in nuclear grade I tumors ($p<1.0x10^{-8}$). When blood WAVE3 expression levels were compared among the patients with different SBR grade tumors, WAVE3 levels were the highest in the blood of patients with SBR3 compared to those patients with SBR1 tumors ($p<2.2e^{-16}$), further confirming the association between WAVE3 expression levels and the tumor SBR grade that we originally derived from the results of the IHC staining analyses (Figure 6B). Next we analyzed the blood WAVE3 levels against the patient's hormone receptor status. We found a significant negative correlation between the blood WAVE3 expression levels and the tumor hormone receptor status, where WAVE3 is significantly highly expressed in the blood of patients with ER-negative (Figure 7A) and PR-negative (Figure 7B) tumors compared to the patients with ER- and PR-positive tumors ($p<2.2x10^{-16}$ and $p<1.1x10^{-14}$, respectively). More importantly, WAVE3 expression levels were found to be significantly highly expressed in the blood of BC patients whose tumors were lacking all three hormone receptors, i.e., ER, PR and Her2neu, also known as TNBC (Figure 7B). This important result supports our initial finding from the established BC cell lines of triple-negative origin (Figure 7C) and also suggests that WAVE3 expression levels in the blood of BC patients can be used as biomarker for the identification of BC patients Triple-Negative tumors. Thus our data identify WAVE3 as a novel biomarker that is specific to the TNBC subtype.

DISCUSSION

Metastatic disease is responsible for the vast majority of cancer-related deaths and has been shown to be controlled by specific genetic events. The complex nature of metastasis from the primary tumor to a distant site involves acquisition of many intermediate phenotypes, which makes the genetics of the overall process very complex. Changes in cancer cell movement, migration and invasion, and its interaction with the extracellular environment are necessary steps in the metastatic cascade. Dissecting the mechanisms controlling these steps is, therefore, vital to our understanding of the metastatic disease and to designing anti-metastatic therapies. Our in vitro and preclinical in vivo studies have identified the WAVE3 gene as a major player in these critical steps that lead to cancer metastasis. The role of WAVE3 as a metastasis promoter gene is supported by the following observations: (*i*) WAVE3 is required and sufficient to drive cancer cell invasion and tumor metastasis; (*ii*) the metastasis promoting activity of WAVE3 is further enhanced by phosphorylation downstream of Abl to regulate key aspects of cancer cell invasion, i.e., invadopodia and MMP activity and (*iii*) WAVE3 expression is regulated by two microRNAs (miRs 200 and 31) which have been established as master regulators of EMT and invasion-metastasis cascade, respectively. Furthermore, the top-10 list of WAVE3-targeting microRNAs contains [33], other than miR31 and members of the miR200 family, miRs 570, 542, 103, 107 and 302, all of which have been found to be deregulated during cancer progression and metastasis [26,33,39–44], therefore, providing further support for the function of WAVE3 as a metastasis-promoter gene.

Our present clinical study further provides findings, from a biologic perspective, that strongly support what has been observed in *in vitro* and pre-clinical *in vivo* studies. Our IHC analyses clearly show that WAVE3 is expressed in the highest amounts in those tumors that possessed

prognostic factors most frequently associated with the development of distant metastasis in breast cancer, namely positive lymph node status and tumor size. (6, 8, 20-23). We did not see an association between WAVE3, ER expression and grade in our tissue samples as we did in our serum samples. This is likely due to the smaller sample size that did not give us adequate power to detect a difference. We originally identified over 200 cases for this study from our database, due to spent tissue blocks, tissue loss from TMAs, missing clinical data we had only 128 evaluable cases with complete tissue and clinical information. However, WAVE3 expression was also significantly increased in the primary tumors of women who subsequently developed distant metastatic disease, compared to women with local or no metastasis. This observation is clinically significant since it supports the use of WAVE3 as a biomarker for early detection and identification of women with breast cancer who may be at risk of progression to more aggressive and therefore incurable distant metastatic disease. Our findings also show a very significant association between WAVE3 expression and both the disease specific survival and the risk of distant recurrence free survival in both the univariate and multivariate analyses (Tables 5 and 6).

Our finding in BC are corroborated by similar findings in prostate cancer, where Fernando et al., reported that WAVE3 score was found to be significantly correlated with advanced human prostate cancer (22), further strengthening the position of WAVE3 as a useful biomarker for cancer progression and metastasis.

The major aim of our second study aimed at validating the findings of the IHC analyses using a much less invasive bioassay, i.e., blood samples collected from the patients during one of their visits to an outpatient facility. Our main hypothesis was that, since WAVE3 expression levels are very low in the normal PBMCs, any quantitative increased levels of WAVE3 in the blood of BC patients must come from the CTC that have escaped the primary tumors a were disseminated in

the circulation. For that end we used blood specimens from 200 patients with different clinical and pathological subtypes of BC. Other rational behind this study is the fact BC is considered a systemic disease since early cancer cell dissemination may occur even in patients with small tumors. Furthermore it has been shown that epithelial cells (Cancer cells) can be identified in the peripheral blood of otherwise metastasis-free patients with stage I and II breast cancer [45–52] and the association between the presence of circulating tumor cells in the blood of patients with metastatic carcinoma and short survival has been confirmed in several studies [45,46,48,49,53]. The detection of circulating tumor cells has, therefore, been proposed, as a method of choice for risk stratification in early breast cancer, in early detection of relapse and in monitoring the response to treatment of metastatic carcinoma [47,49,51,53,54]. On the other hand, it has been shown that adjuvant chemotherapy fails to completely eliminate CTCs [45–51,53], and persistence of CTCs after adjuvant chemotherapy has been associated with a poor clinical outcome in patients with early-stage breast cancer [45–51,53]. These observations strongly suggest that the identification of biomarkers that are specific to CTCs may be of clinical importance to follow in patients with early-stage breast cancer, because they may identify a subgroup of patients who are at high risk of relapse. Moreover, the detection of tumor cells-specific biomarkers in the blood before and after the adjuvant systemic treatment could help to identify those patients who may have a substantial clinical benefit from a 'secondary' adjuvant treatment before the occurrence of overt metastasis. We, therefore, sought propose to evaluate the prognostic value of WAVE3 expression levels in the peripheral blood of women with operable breast cancer.

Our pilot study clearly demonstrated that significantly high levels of WAVE3 can easily be detected in the blood of collected from BC who also developed metastatic disease, compared to

the healthy controls, therefore, demonstrating the proof of principle that increased WAVE3 expression levels can be used as a biomarker for the presence of CTCs in the blood of BC patients. Our subsequent analysis of WAVE3 expression in the blood of 200 BC patients proved that WAVE3 expression levels correlate positively with adverse disease characteristics, namely, tumor nuclear grade, SBR grade and hormone receptors status. The most significant finding of this analysis is the fact that WAVE3 expression levels were significantly higher in the blood of patients with subtype TNBC disease compared to the other BC subtypes ($p<2.2x10^{-16}$), clearly demonstrating that WAVE3 expression levels can distinguish this very aggressive subtype of BC from the other subtypes. Within BC subtypes, those classified as TNBCs exhibit dismal survival rates due to their highly aggressive and metastatic behavior, and to their propensity to rapidly recur [14–20]. Furthermore, the absence of novel therapies capable of specifically targeting this aggressive BC subtype is a direct consequence of the lack of sufficient knowledge about TNBC development and progression, further contributing to the aggressive relapse and dismal survival rates amongst women bearing TNBCs [6,9,10,23,55]. Therefore, the development of rapid and sensitive diagnostic tests capable of detecting developing TNBCs in otherwise seemingly healthy women to be employed in clinical settings is warranted. Together, our data clearly identify WAVE3 as a novel biomarker for the progression and metastasis of breast cancer. More importantly, our data support the use of WAVE3 a specific marker for identification of the most aggressive forms of BC, i.e., the TNBC. Moreover, in an applied clinical setting, the detection of WAVE3 in the blood of BC patients after the completion of an adjuvant systemic treatment could help identify those patients who may have a substantial clinical benefit from a 'secondary' adjuvant treatment before the occurrence of overt metastasis.

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AUTHOR CONTRIBUTIONS

SK designed the study of the study, participated in the design and oversaw the coordination of the study, participated in interpretation of the data drafted the manuscript, KA and BM performed experiments, LR participated in performing the statistical analysis and in the interpretation of the data, TK read the immunohistochemistry, LT and LZ oversaw the statistical analyses and participated in the study design, AG performed the statistical analyses, KSA conceived of the overall concept of the study, participated in writing and preparing the manuscript.

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FIGURE LEGENDS

Figure 1. WAVE3 expression levels positively correlate with the aggressiveness of breast cancer cell lines. (A) Western bolt analysis of protein lysates isolated from the indicated BC cell lines using WAVE3 antibody. Actin was used as a loading control. (B) Representative micrographs of Matrigel invasion of the indicated BC cell lines and quantification of the Matrigel invasion (C). The results are shown as the mean ± s. d. of at least 3 independent assays.

Figure 2. Loss of WAVE3 attenuates the aggressiveness of the BC cells of Triple-negative subtype. (A) Quantitative Real-time RT-PCR of WAVE3 in the indicated BC cell lines after transient transfection with either a control siRNA or WAVE3-specific siRNA. (B) Western blot analysis of protein lysates following the same treatment as in (A). Actin was used a loading control and WAVE2 was used a negative control. (C and D) Matrigel invasion assay after the indicated treatments. Representative micrographs are shown in (C), and the quantitation is shown in (D). The results are shown as the mean ± s. d. of at least 3 independent assays.

Figure 3. WAVE3 expression is increased in the high grade BC. Representative WAVE3 IHC micrographs with matching hematoxylin & eosin of BC tumors. (A & B) mSBR grade 1 with negative staining and (C &D) mSBR grade 3 with diffusely strongly positive (score 300) staining.

Figure 4. Figure 4. WAVE3 expression levels are positively correlated with reduced distant recurrence free survival and with decreased disease specific survival. Kaplan-Meier analysis of (A) Overall survival, (B) Distant recurrence free survival and (C) Disease specific survival. The

WAVE3 score was dichotomized as positive or negative to determine the relative contribution of the WAVE3 score to each of the three disease outcome parameters, respectively. The midpoint of the difference between the 75% CI for the WAVE3 score of patients with overall survival, no distant recurrence or breast cancer related mortality and the 25% CI was (212) was used.

Figure 5. WAVE3 mRNA is highly expressed in the peripheral blood of patients with metastatic breast cancer. (A) Semi-quantitative RT-PCR from total RNA extracted from a mix of 5 million cells of EBV-lin (a PBMC) and MDA-MB-231 (a highly metastatic breast cancer cell line), at the indicated ratios. MDA-MB-231 cells and EBV-Lin cells were used alone as a positive and negative controls, respectively. WAVE3 mRNA could be amplified from the positive control cells (MDA-MB-231), but not form from the white blood cells (EBV-Lin). WAVE3 mRNA could also be amplified from as few as 1 cancer cell in a million blood cells. GAPDH was used as an internal control for the integrity of the RNA and as equal loading control. (B) Semi-quantitative RT-PCR and (C) quantitative real-time RT-PCR analyses of WAVE3 mRNA expression levels in the blood of 10 patients with metastatic BC and 10 healthy controls. MCF10A was used as a control. The graphs were plotted in a logarithmic scale with the average fold change to MCF10A ± s. d. is shown under the respective bar. GAPDH was used an internal normalization control. The results are shown as the mean ± s. d. of at least 3 independent assays.

Figure 6. WAVE3 expression levels in the blood of BC patients correlate positively with the aggressiveness of the primary tumor. WAVE3 expression levels were analyzed by quantitative real-time RT-PCR and plotted against the nuclear grade (A) and the SBR grade (B) of the

primary tumor. WAVE3 RT-PCR values were normalized to GAPDH and plotted as the fold change to MCF10A. The results are shown as the mean ± s. d. of at least 3 independent assays.

Figure 7. WAVE3 expression levels in the blood of BC patients correlate positively with the aggressive TNBC subtype. WAVE3 expression levels were analyzed by quantitative real-time RT-PCR and plotted against the ER (A) and PR (B) hormone receptor status of the primary tumor. In (C) WAVE3 expression levels were compared between the patients with TNBC and other subtypes. WAVE3 RT-PCR values were normalized to GAPDH and plotted as the fold change to MCF10A. The results are shown as the mean ± s. d. of at least 3 independent assays.

TABLES

Table 1. Clinicopathological variables

	mSBR1 (n=64)	mSBR3 (n=64)	p value
Age at Diagnosis (years)	57.3±12.1	56.3±12.9	0.755 [a]
Tumor Size	2.0±1.4 cm	2.4±2.0 cm	0.243 [a]
Lymph Node Status			0.720 [b]
Negative	36 (54.7%)	39 (59.4%)	
Positive	29 (45.3%)	26 (40.6%)	
TNM Stage			1.0 [b]
Stage I	24 (37.5%)	23 (36%)	
Stage II	31 (48.4%)	32 (50%)	
Stage III	9 (14.1%)	9 (14%)	
Her2 Status			<0.001 [b]
Negative	59 (92.2%%)	44 (68.8%)	
Positive	3 (4.7%)	20 (31.2%)	
Frequency of recurrence			<0.001 [b]
All recurrence	1 (1.6%)	17 (26.6%)	
Local recurrence	1 (1.6%)	6 (9.4%)	
Distant recurrence	0 (0%)	11 (17.2%)	
Disease related mortality	0 (0%)	8 (12.5%)	0.003 [b]

[a]Measured as a continuous variable, Mann-Whitney test

[b]Chi-square test

Table 2. Tumor WAVE3 score, benign WAVE3 score and differential WAVE3 compared by risk group

	Median	25-75% quartile	p Value [a]
WAVE3 score			0.001
Tumor	180	100-255	
Benign	65	30-160	
Differential WAVE3 expression			0.714
mSBR1	80	0-160	
mSBR3	75	0-185	

[a]Measured as a continuous variable, Mann-Whitney test

Table 3. Relationship of WAVE3 score to pathologic tumor features

	Median	25-75% quartile	p Value
Lymph Node Status			0.017 [a]
Negative	140	80-210	
Positive	200	135-277	
Stage			0.023 [b]
I	160	100-240	
IIA	120	68-200	
IIB	200	180-300	
IIIA	200	160-240	
IIIB	300	210-300	
IIIC	255	180-285	
Her2 neu Status			0.519 [a]
Negative	160	92-263	
Positive	200	120-240	

[a]Measured as a continuous variable, Mann-Whitney test

[b]Measured as a continuous variable, Kruskal-Wallis test

Table 4. Comparison of WAVE3 score by clinical outcome

	WAVE3 Score	25-75% quartile	p value
Recurrence			0.156 [a]
None	160	100-240	
Any	220	125-293	
Overall Survival			0.028
Surviving	160	100-240	
Disease related mortality	255	220-300	

[a]Measured as a continuous variable, Mann-Whitney test

Table 5. Multivariate analysis for risk of distant recurrence free survival

Variable	Hazard Ratio	95% CI	p value
Age	0.99	(0.94,1.04)	0.683
Tumor size	1.18	(2.02,65.52)	0.317
+ Lymph node status	11.50	(1.77,221.23)	0.006
ER -	42701.96	(0.00,inf)	0.740
Her2 neu +	0.17	(0.03,0.96)	0.045
WAVE3 Score >212	3.88	(0.78,19.33)	0.0013

Table 6. Multivariate analysis of disease specific survival

Variable	Hazard Ratio	95% CI	p value
Age	1.00	(0.94,1.06)	0.9766
Tumor size	0.96	(0.73,1.25)	0.7350
+ Lymph node status	19.78	(1.77,221.23)	0.0154
ER -	34596.99	(0.00, Inf)	0.7678
Her2 neu +	0.31	(0.05,1.97)	0.2164
WAVE3 Score >212	15.50	(1.77,135.71)	0.0133

Figure 1

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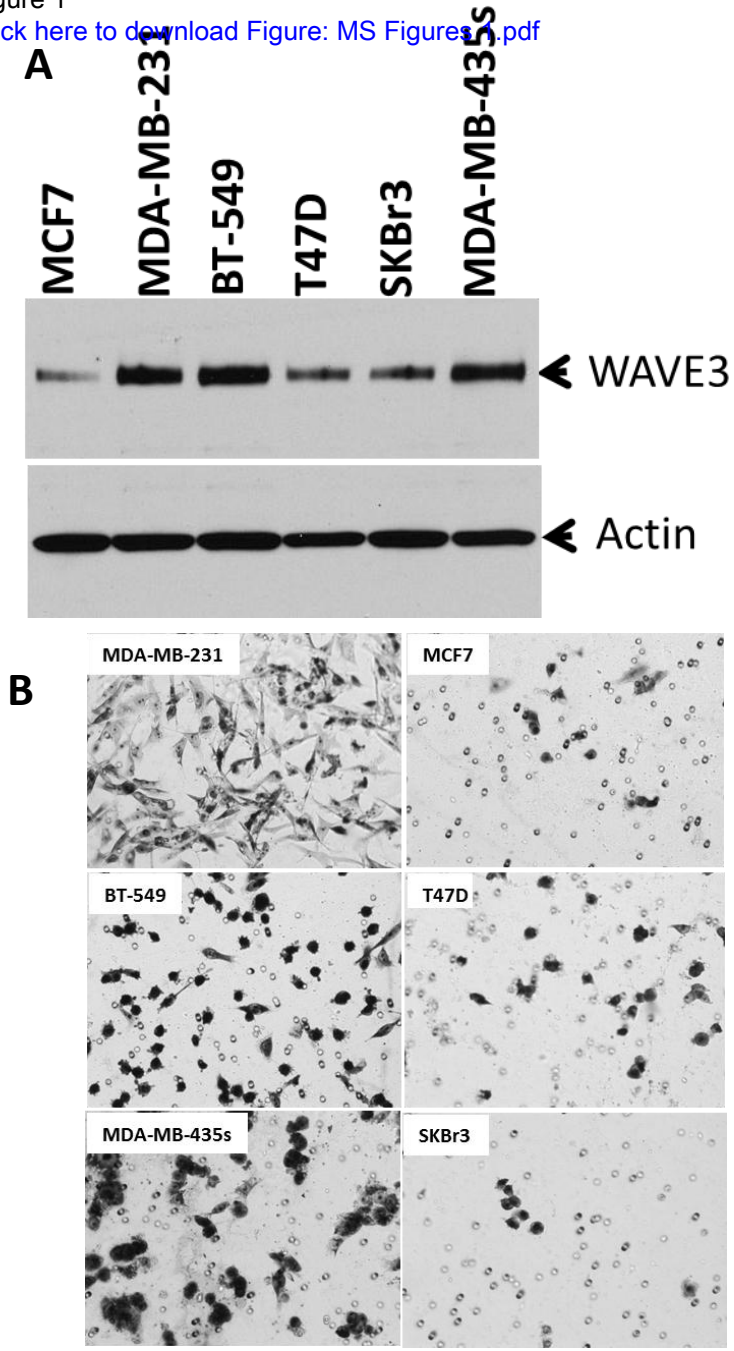

Figure 1

Figure 2

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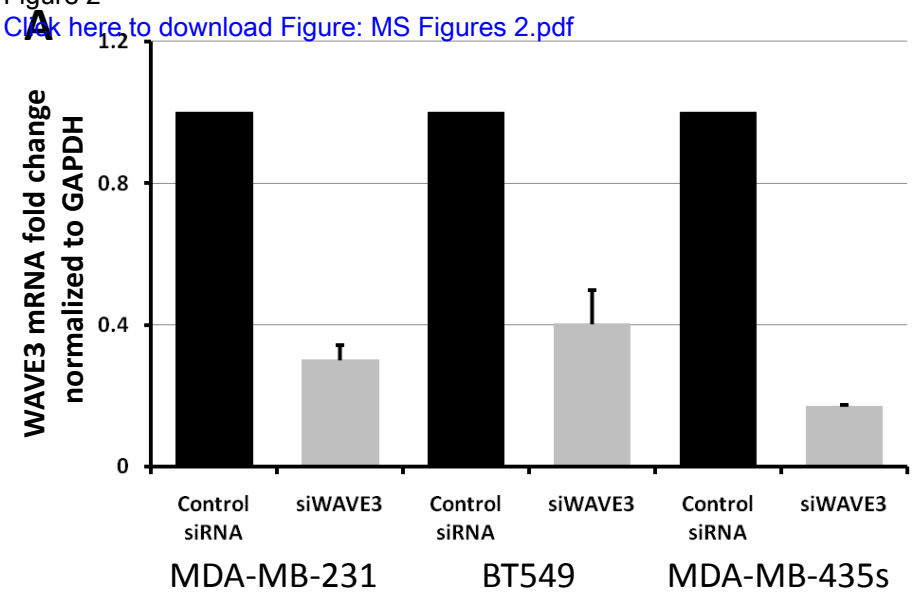

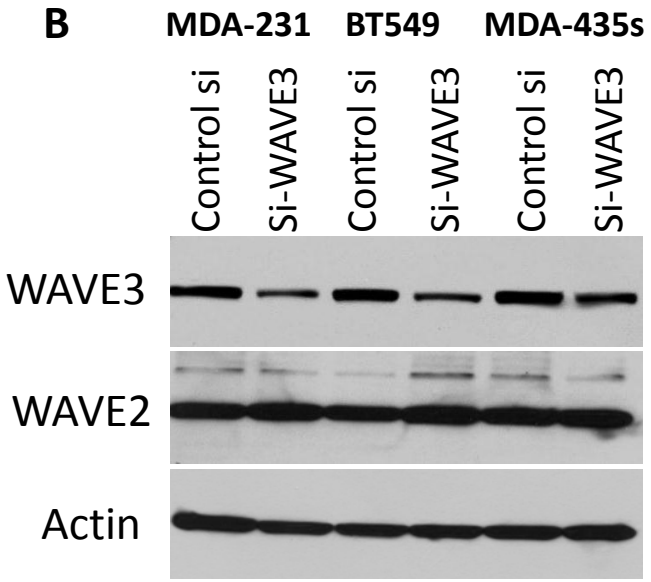

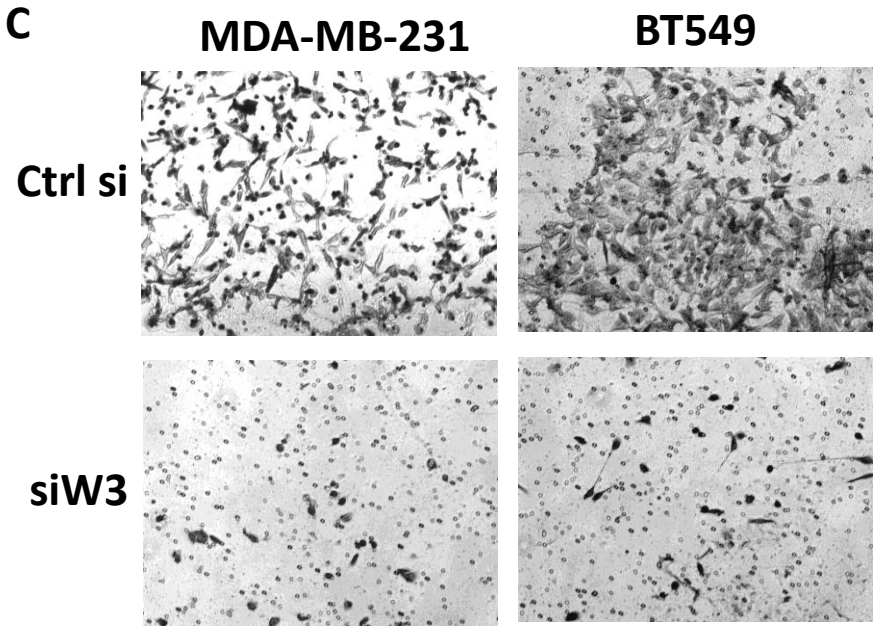

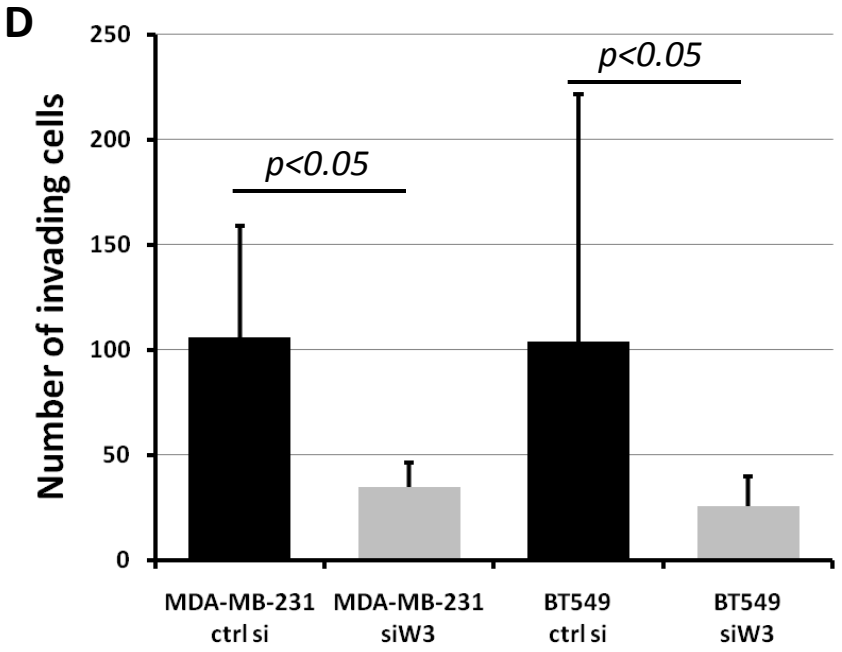

Figure 2

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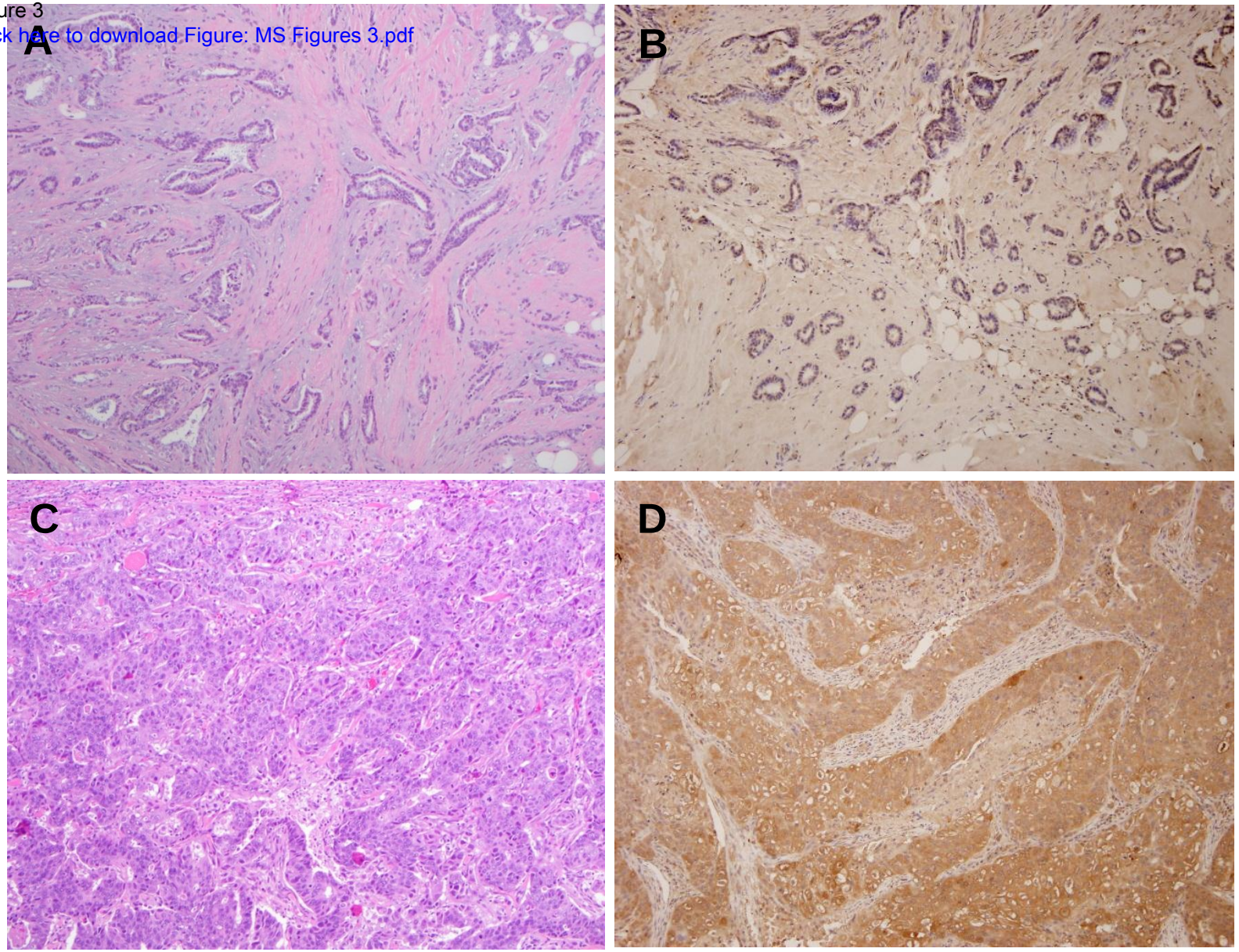

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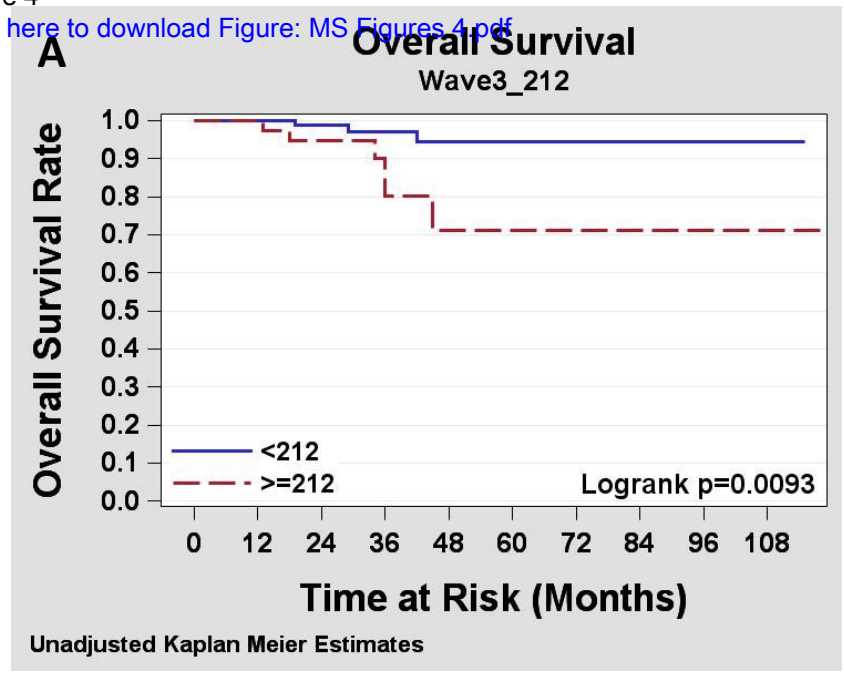

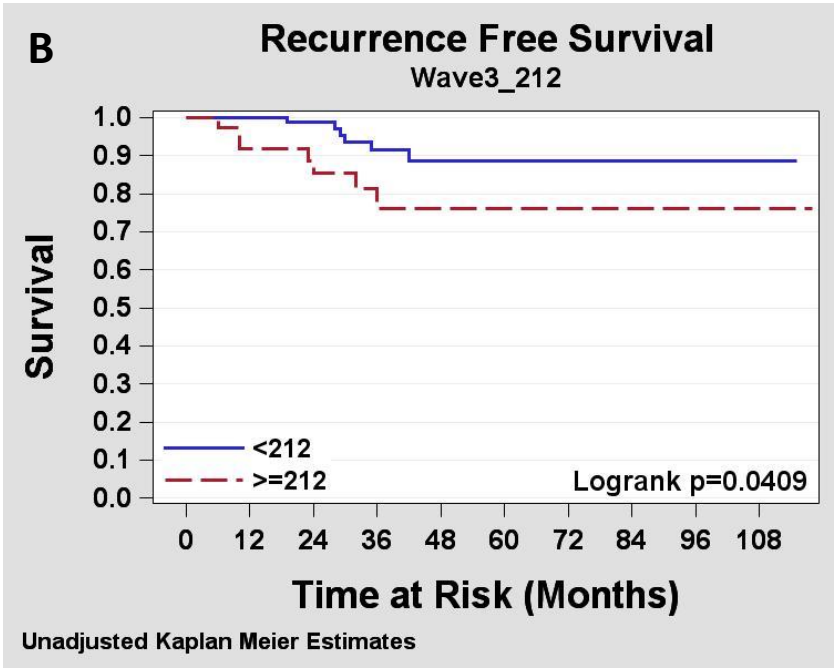

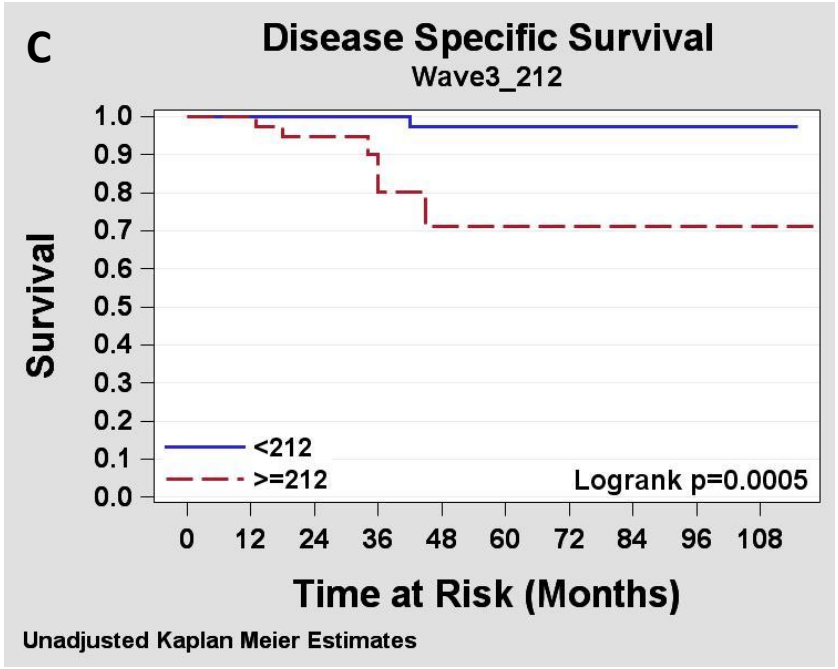

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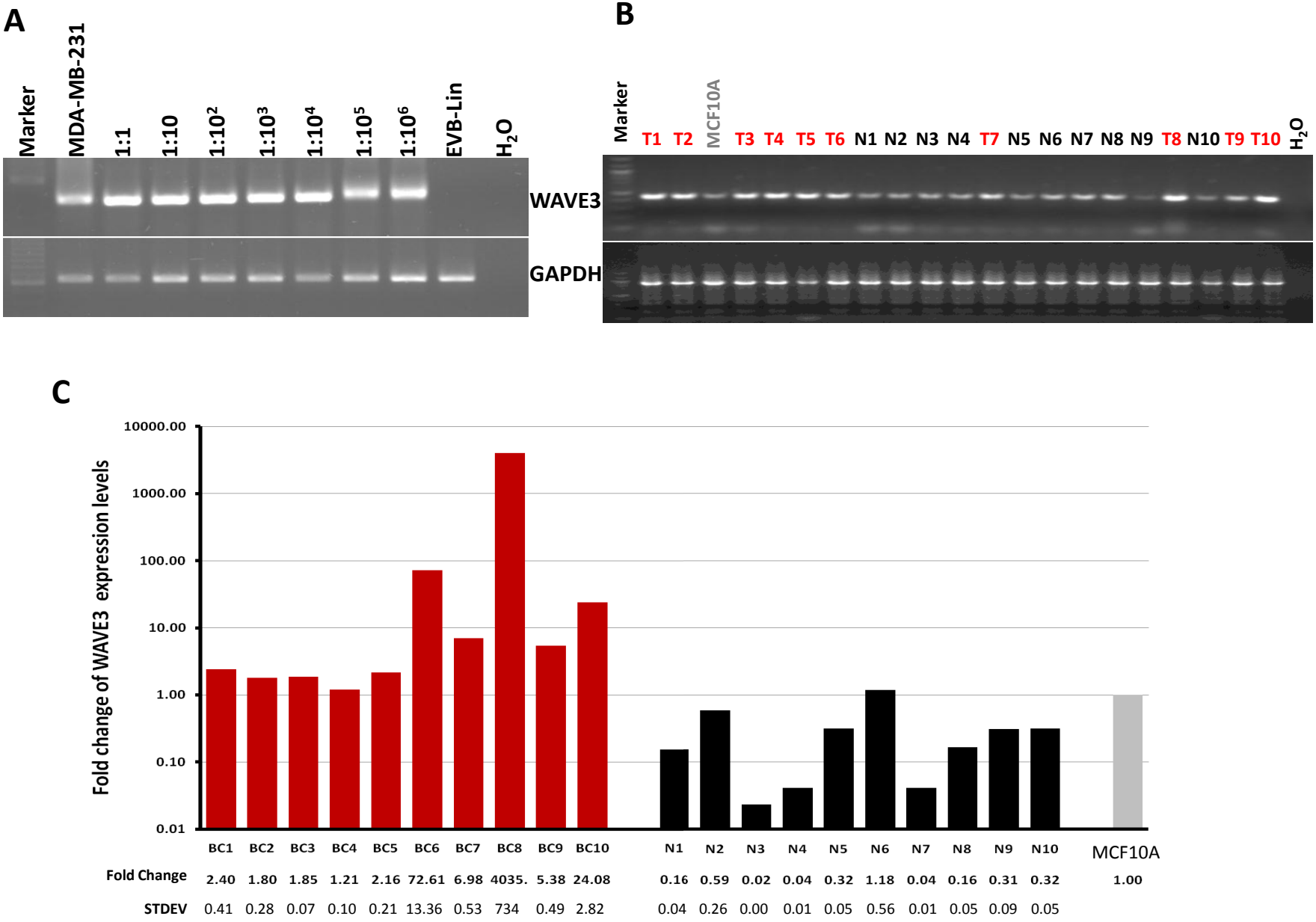

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Figure 6
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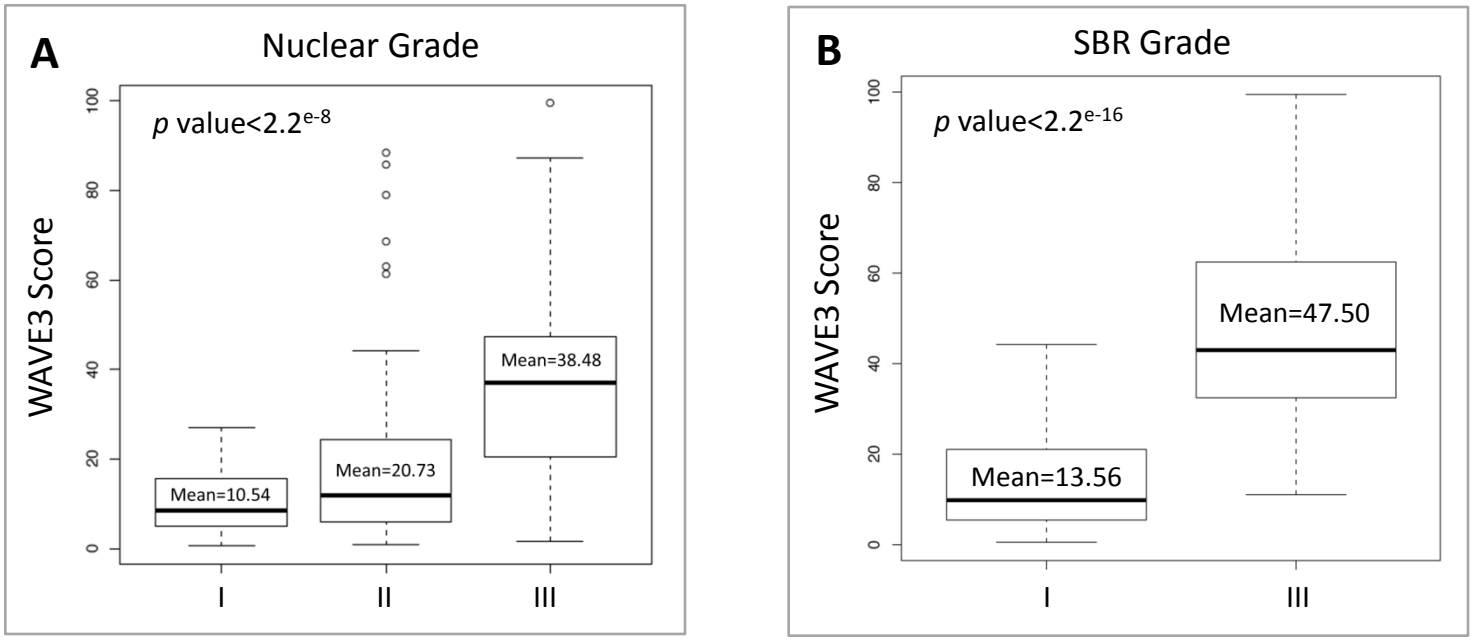

Figure 6

Figure 7

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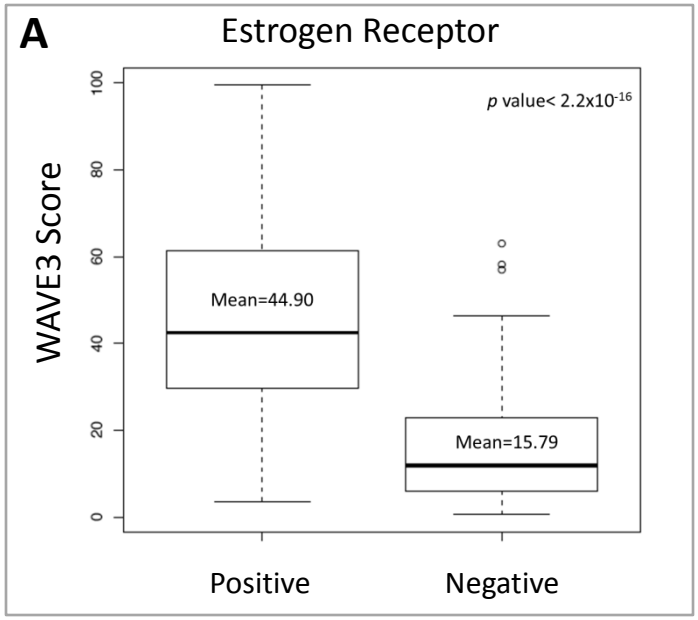

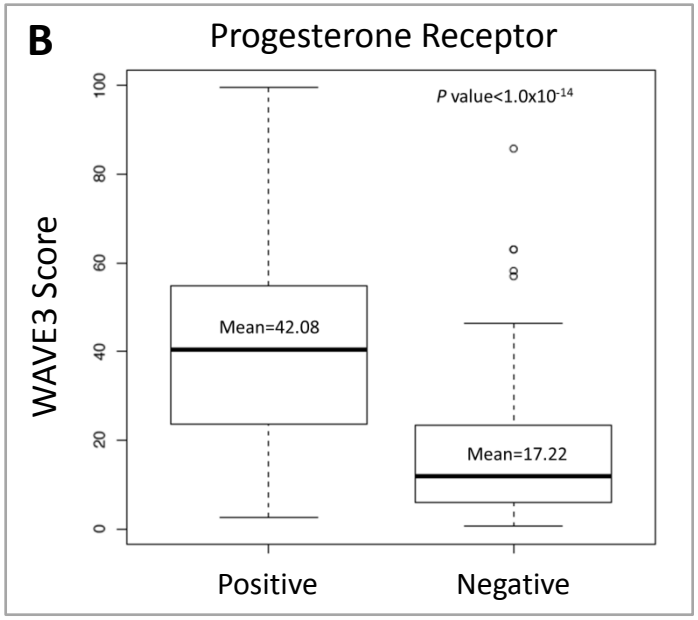

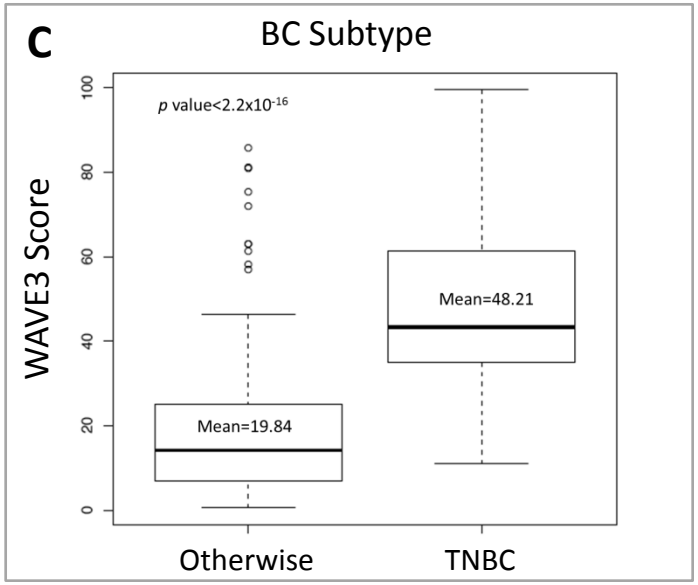

Figure 7

Supporting Information Table S1

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Supporting Information Table S2

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Supporting Information Table S3

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